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Effects of mutagenesis of residue 221 on the properties of bacterial and mitochondrial elongation factor EF-Tu ☆

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Abstract

During protein biosynthesis, elongation factor Tu (EF-Tu) delivers aminoacyl-tRNA (aa-tRNA) to the A-site of ribosomes. This factor is highly conserved throughout evolution. However, several key residues differ between bacterial and mammalian mitochondrial EF-Tu (EF-Tu_{mt}). One such residue is Ser221 (*Escherichia coli* numbering). This residue is conserved as a Ser or Thr in the bacterial factors but is present as Pro269 in EF-Tu_{mt}. Pro269 reorients the loop containing this residue and shifts the adjoining β-strand in EF-Tu_{mt} compared to that of *E. coli* EF-Tu potentially altering the binding pocket for the acceptor stem of the aa-tRNA. Pro269 was mutated to a serine residue (P269S) in EF-Tu_{mt}. For comparison, the complementary mutation was created at Ser221 in *E. coli* EF-Tu (S221P). The *E. coli* EF-Tu S221P variant is poorly expressed in *E. coli* and the majority of the molecules fail to fold into an active conformation. In contrast, EF-Tu_{mt} P269S is expressed to a high level in *E. coli*. When corrected for the percentage of active molecules, both variants function as effectively as their respective wild-type factors in ternary complex formation using *E. coli* Phe-$tRNA^{Phe}$ and Cys-$tRNA^{Cys}$. They are also active in A-site binding and in vitro translation assays with *E. coli* Phe-$tRNA^{Phe}$. In addition, both variants are as active as their respective wild-type factors in ternary complex formation, A-site binding and in vitro translation assays using mitochondrial Phe-$tRNA^{Phe}$.

Keywords: Mitochondria; Bacteria; Protein synthesis; Elongation; Elongation factor Tu; Mutagenesis

1. Introduction

During protein biosynthesis, elongation factor Tu (EF-Tu), in its active GTP conformation, binds aminoacyl-tRNA (aa-tRNA) and promotes its binding to the A-site of the ribosome [1]. Upon positive codon/anticodon interactions, GTP is hydrolyzed to GDP and the inactive EF-Tu·GDP complex dissociates from the ribosome, leaving the aa-tRNA in the A-site. After dissociation, the guanine nucleotide exchange factor, elongation factor Ts (EF-Ts), displaces GDP and binds EF-Tu. EF-Ts is then displaced by GTP, thus reactivating EF-Tu and allowing the formation of another ternary complex.

Mammalian mitochondria have a translational system with both similarities and differences to the prokaryotic system. Mitochondrial EF-Tu (EF-Tu_{mt}) functions in translation in a manner analogous to the bacterial factors. However, the binding constants that govern the interactions between EF-Tu and its ligands differ significantly between *Escherichia coli* EF-Tu and EF-Tu_{mt} [2]. The only structure of EF-Tu_{mt} currently available is that of bovine EF-Tu_{mt} in complex with GDP which shows that the structure of the mitochondrial factor is very similar to that of its prokaryotic counterparts [3].

One of the most unusual features of the mammalian mitochondrial translational system is its tRNAs. The primary sequences of most tRNAs contain conserved or semi-conserved residues that enable the tRNAs to fold into stable "L" shaped structures. Canonical tRNAs also contain a high G–C content in their stem regions to improve their stabilities [4]. Mitochondrial tRNAs lack a number of the invariant and semi-invariant residues found in other tRNAs. Furthermore, they have a high A–U content in their stem regions and are often significantly shorter than canonical tRNAs, having as few as 59 nucleotides compared to 75 or more in other tRNAs [3,4]. No direct structural information is available on these tRNAs, although chemical probing and NMR analysis suggest that they do form a basic L-shape [5].

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In contrast, transient electric birefringence measurements suggest that they may look more like a boomerang than an L [6]. Both *E. coli* EF-Tu and EF-Tu_{mt} are able to form ternary complexes with mitochondrial aa-tRNAs. However, *E. coli* EF-Tu is significantly less active than EF-Tu_{mt} in delivering them to the A-site of the ribosome [7]. The fundamental basis for this difference is not known.

The crystal structure of *E. coli* EF-Tu indicates that it is composed of three domains [8–10]. All three domains of EF-Tu are involved in binding aa-tRNA. The aminoacyl group of the aa-tRNA is bound in a pocket created between Domains I and II. Domain II is mainly responsible for binding the 3′ end of the tRNA. A portion of the acceptor stem and the 5′ end of the aa-tRNA are bound in a pocket formed by all three domains of EF-Tu. The extended acceptor-TψC helix of the aa-tRNA lies across Domain III [1,11].

Most of the residues that create the aa-tRNA binding pocket are highly conserved in the EF-Tu family. One of these is Ser221 (*E. coli* numbering) in Domain II. In *Thermus aquaticus* EF-Tu, this residue is part of the binding pocket for the amino acid of the aa-tRNA. It is also present in one of the two loops that form the binding pocket for the terminal adenosine of the aa-tRNA [8]. In the ternary complex with Cys-$tRNA^{Cys}$, it actually forms a hydrogen bond with C75 [8,9]. Strikingly, this position is occupied by a Pro (Pro269) in mammalian EF-Tu_{mt}. In the present work, the effects of mutating Pro269 in EF-Tu_{mt} to Ser and of converting Ser221 in *E. coli* EF-Tu to Pro are explored.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from New England Biolabs. Superase·In RNase inhibitor was purchased from Ambion. Mitochondrial EF-G [12,13] and crude mitochondrial ribosomes [14] were purified from bovine liver. Purified mitochondrial ribosomes were prepared from crude ribosomes as described [15]. *E. coli* ribosomes were prepared from *E. coli* W [7,12]. *E. coli* and mitochondrial EF-Ts were expressed in *E. coli* as histidine-tagged proteins and purified on Ni-NTA resin [12,16]. Crude *E. coli* tRNA was purchased from Boehringer Mannheim. [^{14}C]Phe-$tRNA^{Phe}$ and [^{35}S]Cys-$tRNA^{Cys}$ were prepared from *E. coli* tRNA as described [17]. Bovine mitochondrial tRNA was isolated from purified bovine mitoplasts (mitochondria from which the outer membrane has been removed) using the Qiagen RNA/DNA Maxi Kit. Mitochondrial $tRNA^{Phe}$ was aminoacylated with [^{14}C]Phe using human mitochondrial Phe-tRNA synthetase [18].

2.2. Cloning *E. coli* EF-Tu S221P and EF-Tu_{mt} P269S

A gene encoding a His-tagged variant of *E. coli* EF-Tu cloned into pET-24c(+) [16] was used as a template for the mutagenesis of Ser221 to Pro using the Stratagene QuikChange Site Directed Mutagenesis protocol. Synthetic oligonucleotides 5′-gtattctccatcccaggtcgtggtaccgttgttaccgg-3′ and 5′-taccacgacctgggatggagaatacgtc-3′ were used as forward and reverse primers, respectively. The P269S variant of EF-Tu_{mt} was prepared using similar procedures except the template used was the coding region of the mature form of EF-Tu_{mt} inserted into pET-24c(+) [19]. The forward and reverse oligonucleotides used to replace Pro269 with Ser were 5′-gtactcgatctccggccggggcacagtggtgacaggcacg-3′ and 5′-tgccccggccggagatcgagtac-3′, respectively.

2.3. Protein expression and purification

The plasmids encoding wild-type *E. coli* EF-Tu, *E. coli* EF-Tu S221P, wild-type EF-Tu_{mt} and EF-Tu_{mt} P269S were transformed into *E. coli* BL21(DE3) cells for expression. Cultures were grown in 2xYT media containing 50 μM kanamycin at 37 °C to a cell density of 0.6–0.9 A_{600}. Expression was induced by the addition of 50 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 4 h. The proteins were purified using Ni-NTA columns as described except all buffers contained 50 μM GDP [19]. Alternatively, cells were resuspended in 40 ml lysis solution (50 mM Tris–HCl (pH 7.6), 40 mM KCl, 7 mM $MgCl_2$, 10% glycerol, 7 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.03% egg-white lysozyme, 0.1% Triton X-100 and 5 μg/ml DNase 1), incubated on ice for 10 min and sonicated for 9 min (1 s bursts with 4 s cooling) on ice at an output of 60 W. Extracts were centrifuged at 27,000 × *g* for 30 min at 4 °C followed by ultracentrifugation at 100,000 × *g* for 1 h at 4 °C. The factors were purified on Ni-NTA agarose using buffers containing 50 μM GDP as described [19]. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard [20].

2.4. Time course of induction

E. coli BL21(DE3) cell cultures (30 ml) carrying the plasmids encoding either wild-type *E. coli* EF-Tu or *E. coli* EF-Tu S221P were grown to mid-log phase at 37 °C and induced with 50 μM IPTG for 0, 0.5, 1, 2, 3, 4 and 5 h. At each time point indicated, a 1 ml aliquot was removed from the cell culture and the A_{600} was measured. Cells from a portion of this aliquot (0.8 ml) were harvested by centrifugation at 16,000 × *g* for 1 min at 4 °C. Cell membranes were lysed and possible inclusion bodies were solubilized in 0.2 ml lysis buffer containing 8 M urea, 0.1 M sodium phosphate (pH 8.0) and 0.01 M Tris–HCl (pH 8.0). Following clarification of the supernatant by centrifugation at 16,000 × *g* for 10 min at 4 °C, the extracts were incubated (with slight agitation) with 0.05 ml of Ni-NTA resin (50% slurry) at 25 °C for 0.5 h. The Ni-NTA resin was collected at 13,800 × *g* for 1 min at 4 °C and washed three times with 1 ml wash buffer containing 8 M urea, 0.1 M sodium phosphate (pH 6.4) and 0.01 M Tris–HCl (pH 6.3). Protein bound to the resin was eluted with wash

buffer (0.02 ml) containing 0.1 M EDTA following incubation (with slight agitation) at 25 °C for 2 min. The resin was pelleted at 16,000 × *g* for 1 min at 4 °C and the supernatants were subjected to SDS-polyacrylamide gel electrophoresis on 10% gels.

2.5. In vitro translation

Poly(U)-directed polymerization of [^{14}C]Phe was used to assay the function of wild-type and variant EF-Tu as described [7,12,13]. Reaction mixtures (100 μl) contained 50 mM Tris–HCl (pH 7.8), 1 mM dithiothreitol, 0.1 mM spermine, 80 mM KCl, 6 mM $MgCl_2$, 2.5 mM phospho*enol*pyruvate, 0.5 units pyruvate kinase, 0.5 mM GTP, 0.125 mg/ml poly(U), 50 units Superase·In, 0.35 μM *E. coli* [^{14}C]Phe-tRNA^{Phe}, 24 units (about 0.01 μM) of *E. coli* EF-G, 0.24 μM *E. coli* ribosomes and 1–10 nM EF-Tu in the presence EF-Ts at a 1:1 (EF-Tu$_{mt}$:EF-Ts$_{mt}$) or 5:1 (*E. coli* EF-Tu:*E. coli* EF-Ts) molar ratio. The amount of [^{14}C]Phe polymerized in each assay was corrected for the retention of radioactivity on the filter in a minus EF-Tu control (approximately 0.5 pmol).

In vitro translation assays using mitochondrial ribosomes were carried out as described above with the following modifications. Reaction mixtures (100 μl) contained 50 mM Tris–HCl (pH 7.8), 1 mM dithiothreitol, 0.1 mM spermine, 40 mM KCl, 7.5 mM $MgCl_2$, 2.5 mM phospho*enol*pyruvate, 0.5 units pyruvate kinase, 0.5 mM GTP, 0.125 mg/ml poly(U), 50 units Superase·In, 0.1 μM mitochondrial [^{14}C]Phe-tRNA^{Phe}, 23 units (about 2 nM) mitochondrial EF-G, 0.32 μM purified mitochondrial ribosomes and 0.015–0.025 μM EF-Tu in the presence of EF-Ts at a 1:1 (EF-Tu$_{mt}$:EF-Ts$_{mt}$) or 5:1 (*E. coli* EF-Tu:*E. coli* EF-Ts) molar ratio [12,13].

2.6. Ternary complex formation

Ternary complex formation was assayed by examining the ability of EF-Tu to protect [^{14}C]Phe-tRNA^{Phe} or [^{35}S]Cys-tRNA^{Cys} from hydrolysis by RNase A [12]. EF-Tu (0.05–0.4 μM) complexed with 50 μM GDP was incubated with 2.5 mM phospho*enol*pyruvate, 0.5 units pyruvate kinase, 0.5 mM GTP and 0.24 μM *E. coli* [^{14}C]Phe-tRNA^{Phe}, 0.28 μM *E. coli* Cys-tRNA^{Cys} or 0.1 μM mitochondrial [^{14}C]Phe-tRNA^{Phe} in a 50 μl reaction mixture containing 50 mM Tris–HCl (pH 7.8), 1 mM dithiothreitol, 68 mM KCl and 6.7 mM $MgCl_2$. After incubation at 0 °C for 15 min, 10 μg RNase A was added to digest the free aa-tRNA. Following an additional 30 s incubation at 0 °C the reaction was terminated by the addition of cold 5% trichloroacetic acid followed by a 10 min incubation on ice. The precipitate was collected on a nitrocellulose membrane and the amount of [^{35}S]Cys-tRNA^{Cys} or [^{14}C]Phe-tRNA^{Phe} remaining was determined by scintillation counting. The values obtained are corrected for the amount of radiolabel retained on the filter in a minus EF-Tu control (approximately 0.5 pmol for *E. coli* [^{35}S]Cys-tRNA^{Cys}, less than 0.2 pmol for *E. coli* [^{14}C]Phe-tRNA^{Phe} and less than 0.1 pmol for mitochondrial [^{14}C]Phe-tRNA^{Phe}) [21,22].

The percentage of EF-Tu molecules active in forming ternary complexes with GTP and *E. coli* [^{14}C]Phe-tRNA^{Phe} was determined by a method similar to that above [23]. In these assays, the concentration of EF-Tu was held constant at 0.2 μM while the concentration of *E. coli* [^{14}C]Phe-tRNA^{Phe} was increased to saturating levels (1 μM). The percentage of EF-Tu active in ternary complex formation was determined from the point at which the factor was saturated with tRNA after correcting for the background (the amount of label precipitated in a minus EF-Tu control at each aa-tRNA concentration, 0.2–0.4 pmol). Unless otherwise indicated, input values of EF-Tu used in all assays are corrected for the percentage of EF-Tu molecules active in ternary complex formation.

2.7. GDP-binding

The percentage of variant and wild-type *E. coli* EF-Tu active in binding GDP was determined as described [12]. EF-Tu (0.01–0.4 μM) was incubated with 11.2–19 μM [^{3}H]GDP. The labeled EF-Tu·GDP complex was detected using a filter-binding assay [12]. The percentage of EF-Tu active in binding GDP was determined from the point at which the factor was saturated with GDP after correcting for the background (retention of radiolabeled GDP in a minus EF-Tu control, less than 0.1 pmol).

2.8. A-site binding

The ability of wild-type and variant EF-Tu to deliver Phe-tRNA^{Phe} to the A-site of the ribosome was assayed basically as described [7]. Reaction mixtures (100 μl) contained 50 mM Tris–HCl (pH 7.8), 1 mM dithiothreitol, 0.1 mM spermine, 80 mM KCl, 6 mM $MgCl_2$, 0.5 mM guanosine 5′ -[β,γ-imido]triphosphate (GDPNP), 0.125 mg/ml poly(U), 50 units Superase·In, 0.40 μM *E. coli* ribosomes, 0.28 μM *E. coli* EF-Tu or EF-Tu$_{mt}$ and 0.03–0.09 μM mitochondrial [^{14}C]Phe-tRNA^{Phe}. The quantity of [^{14}C]Phe-tRNA bound to the ribosomes was determined using a filter-binding assay and measured with a liquid scintillation counter. All values are corrected for the amount of radiolabel retained on the filter in the absence of EF-Tu (about 0.8 pmol).

3. Results

3.1. Analysis of the important residues in Domain II of EF-Tu

Domain II of EF-Tu plays a crucial role in the formation of the ternary complex and in the delivery of

the aa-tRNA to the ribosome. Most of the residues in Domain II that contact the aa-tRNA are highly conserved between prokaryotic and mitochondrial EF-Tu (Fig. 1). However, there are a few notable exceptions. For example, the residue at position 221 is Ser or Thr in prokaryotic EF-Tu. However, there is a striking conservation of Pro at this position in mammalian EF-Tu_{mt} including the factors from bovine, human and mouse species (Fig. 1). Since Pro can have significant structural effects on the polypeptide backbone, its presence in mammalian EF-Tu_{mt} could affect its properties, particularly with respect to its ability to promote the use of mitochondrial aa-tRNAs.

According to the crystal structures of EF-Tu_{mt} and *E. coli* EF-Tu, residue 221 lies in a loop region on the surface of Domain II [3,24]. This loop region plays an important role in the formation of the binding pocket for the acceptor stem of the aa-tRNA. This loop has also been implicated in the binding of EF-Tu to ribosomes and on its GTPase activity based on the observation that an *E. coli* EF-Tu G222D variant is unable to hydrolyze GTP on the ribosome and cannot carry out polypeptide synthesis [25]. Using the Topological Comparison Program (TOP), the tertiary structure of Domain II of EF-Tu_{mt} complexed with GDP was superimposed onto that of *E. coli* EF-Tu (Fig. 2A) [26]. Superposition of the β-sheets on the N- and C-terminal sides of the loop containing residue 221 (β-sheets b and c, respectively) clearly indicates that the conformation of the loop is altered by the presence of the Pro residue in EF-Tu_{mt}.

The loop connecting β-sheets b and c (loop b/c) is composed of four residues, residues 221–224. *E. coli* EF-Tu contains a significant twist in loop b/c originating at residue 221 (Fig. 2A). Due to this bend in loop b/c, residue 223 is oriented towards the loop connecting β-sheets j and k (loop j/k) and loop b/c itself is angled inwards towards β-sheets g and j. Conversely, the residues corresponding to positions 221 and 223 in EF-Tu_{mt} (269 and 271, respectively) lie in the same plane and are oriented farther towards the loop connecting β-sheets g and h (loop g/h) than towards loop j/k. In addition, loop b/c of EF-Tu_{mt} is directed outward, away from β-sheets g and j and the remainder of Domain II. It is important to note that the backbone of Domain II remains unchanged in the GDP, GDPNP and ternary complex structures of *T. aquaticus* EF-Tu. Thus, the structural differences observed in Domain II of the *E. coli* and mitochondrial EF-Tu·GDP complexes listed above are expected to be the same in EF-Tu complexed with GTP and aa-tRNA. These structural differences are predicted to narrow the binding pocket for the terminal adenosine of the aa-tRNA formed by loops b/c and g/h in EF-Tu_{mt}.

The altered orientation of the loop outlined above propagates into portions of the adjoining β-sheets. The

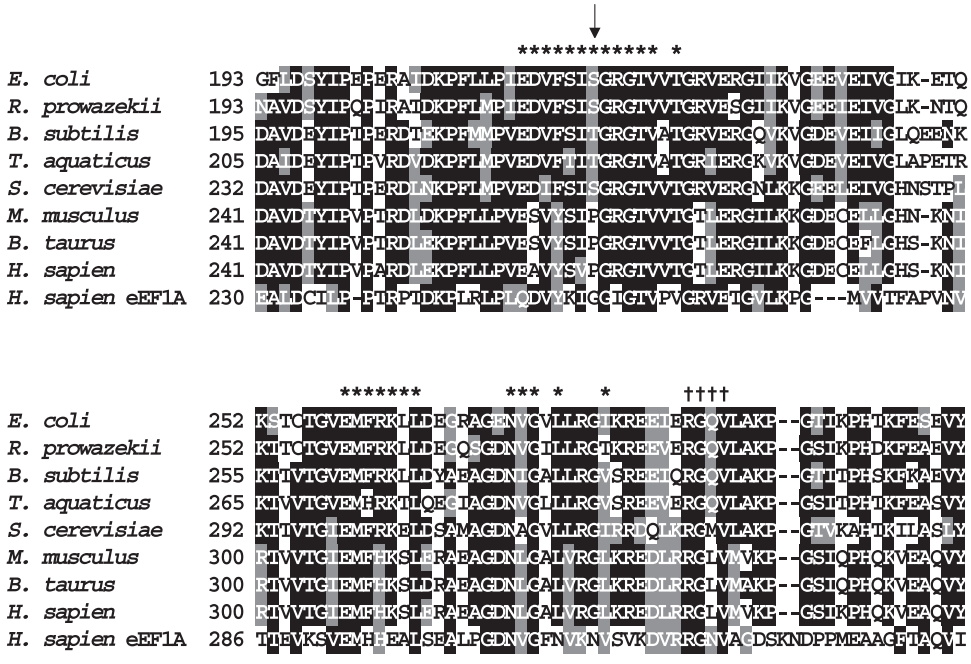

Fig. 1. Primary sequence alignment of Domain II of EF-Tu and eEF1A: the primary sequences of EF-Tu_{mt} were aligned with those from several prokaryotes and that of human eEF1A using the ClustalW program in Biology Workbench. The arrow indicates the residue of interest. The * symbols specify residues involved in binding the 3′ acceptor stem region of the aa-tRNA. The † symbols identify residues involved in binding the 5′ end of the aa-tRNA. The accession numbers for each factor are as follows: *Escherichia coli* EF-Tu (*E. coli*)—P02290; *Rickettsia prowazekii* EF-Tu (*R. prowazekii*)—AJ235272; *Bacillus subtilis* EF-Tu (*B. subtilis*)—P33166; *Thermus aquaticus* EF-Tu (*T. aquaticus*)—S29293; *Saccharomyces cerevisiae* EF-Tu_{mt} (*S. cerevisiae*)—K00428; *Mus musculus* EF-Tu_{mt} (*M. musculus*)—XM_133763; *Bos bovis* EF-Tu_{mt} (*B. bovis*)—P49410; *Homo sapiens* EF-Tu_{mt} (*H. sapiens*)—BC010041; *H. sapiens* cytoplasmic eEF1A (*H. sapiens* eEF1A)—P04720.

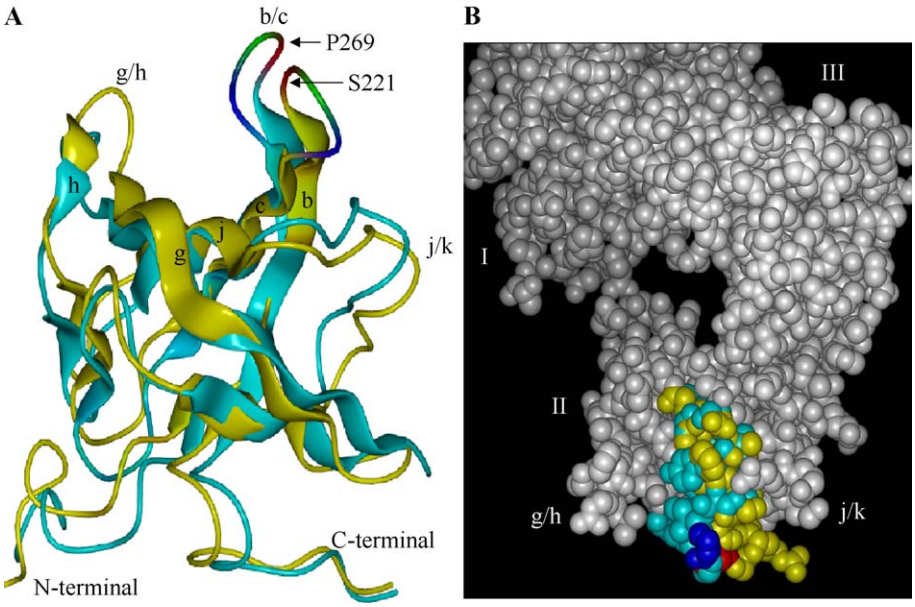

Fig. 2. Effect of the presence of Pro at position 221 on the orientation of the loop in EF-Tu$_{mt}$: (A) Domain II of *E. coli* EF-Tu and EF-Tu$_{mt}$ were superimposed using the Topological Comparison Program (TOP) [26]. The structures were viewed using the Dino Visualizing Structural Biology (2002) (http://www.dino3d.org) and the Persistence of Vision Raytracer (POV-Ray) MegaPov 0.7 rendering http://www.povray.org) software programs. Residue 221 (269 in EF-Tu$_{mt}$) is in red, 222 (270 in EF-Tu$_{mt}$) in green and 223 (271 in EF-Tu$_{mt}$) in blue. *E. coli* EF-Tu is colored yellow and EF-Tu$_{mt}$ is cyan. Loop regions and β-sheets of interest are alphabetized. The coordinates were obtained as PDB# 1EFC for *E. coli* EF-Tu and as PDB# 1D2E for EF-Tu$_{mt}$. (B) Using TOP [26], the GDP-bound structure of *E. coli* EF-Tu was superimposed onto that of EF-Tu$_{mt}$. The PDB coordinates of the loop region b/c and surrounding β-sheets b and c of EF-Tu$_{mt}$ were spliced into the coordinates of *E. coli* EF-Tu and viewed using the Insight II Molecular Modeling Package program http://www.accelrys.com). The resulting structure of *E. coli* EF-Tu.GDP contains loop b/c and β-sheets b and c from both *E. coli* EF-Tu (yellow) and EF-Tu$_{mt}$ (cyan) to illustrate the change in orientation of this loop. Residue 221 of *E. coli* EF-Tu is depicted in red and the corresponding residue 269 of EF-Tu$_{mt}$ in blue.

residues in β-sheets b and c near the loop in *E. coli* EF-Tu are not aligned with the corresponding residues of EF-Tu$_{mt}$. To illustrate the altered orientation of loop b/c and the adjacent residues in EF-Tu$_{mt}$, loop b/c from EF-Tu$_{mt}$ was incorporated into the structure of *E. coli* EF-Tu. As indicated in Fig. 2B, the Pro in loop b/c of EF-Tu$_{mt}$ alters the position of the loop and of the residues in the adjoining β-sheets. The net effect is a change in the spatial arrangement of the region surrounding loop b/c in EF-Tu$_{mt}$ compared to *E. coli* EF-Tu. Since this region is involved in the binding of the 3′ end of the aa-tRNA, the presence of the Pro could have a significant effect on the interaction of EF-Tu with aa-tRNAs.

3.2. Expression and purification of E. coli EF-Tu s221p and EF-Tu$_{mt}$ P269S

Site-directed mutagenesis was performed on Pro269 of EF-Tu$_{mt}$ to mutate it to Ser and the complementary mutation was created on the corresponding Ser221 residue of *E. coli* EF-Tu. The EF-Tu$_{mt}$ P269S and *E. coli* EF-Tu S221P variants were over-produced in soluble form as His-tagged proteins in *E. coli* and purified to near homogeneity. Analysis of the purified proteins using the Bradford method and SDS-polyacrylamide gel electrophoresis indicates that the expression level of EF-Tu$_{mt}$ P269S is approximately 40% of wild-type EF-Tu$_{mt}$ (data not shown). However, a very low yield of the purified *E. coli* EF-Tu S221P variant (approximately 10%) is obtained compared to that of wild-type *E. coli* EF-Tu.

To determine if *E. coli* EF-Tu S221P is being trapped in inclusion bodies, accounting for the low yield, aliquots of cell cultures were extracted under denaturing conditions at various times following induction. The wild-type and S221P variant of *E. coli* EF-Tu were each subjected to purification by Ni-NTA chromatography in 8 M urea and the amount of protein obtained was analyzed by SDS-PAGE (data not shown). Under conditions in which wild-type *E. coli* EF-Tu is easily detected, the yield of *E. coli* EF-Tu S221P is so low that it cannot be detected by Coomassie staining. Thus, chemical lysis of cells indicates that the S221P variant does not get trapped in inclusion bodies. The low yield obtained suggests that a considerable proportion of the S221P variant does not fold properly and is degraded rapidly in the cell.

To determine if expression of *E. coli* EF-Tu S221P is toxic to cells, contributing to the low yields, growth of *E. coli* BL21(DE3) cells carrying the plasmids coding for either wild-type or S221P *E. coli* EF-Tu was monitored before and during expression (data not shown). The cells carrying the *E. coli* EF-Tu S221P clone display a similar growth profile as those containing the wild-type *E. coli* EF-Tu clone indicating that expression of the variant factor is not toxic to cells.

3.3. Activities of *E. coli* EF-Tu S221P and EF-Tu$_{mt}$ P269S

The abilities of *E. coli* EF-Tu S221P and EF-Tu$_{mt}$ P269S to sustain poly(U)-directed polymerization were assayed and compared to their respective wild-type factors. Initial observations indicated that the activity of the P269S variant of EF-Tu$_{mt}$ is similar to (although slightly lower than) that of the wild-type EF-Tu$_{mt}$ (Fig. 3). However, the S221P variant of *E. coli* EF-Tu shows a drastic decrease in poly(U)-directed polymerization compared to wild-type *E. coli* EF-Tu (Fig. 3A). These initial assays suggest that the S221P mutation significantly decreases the activity of *E. coli* EF-Tu in translation.

It has been observed that purified EF-Tu is not fully active in its ability to bind guanine nucleotides or to form ternary complexes with aa-tRNA [23]. The decreased activity of the S221P variant could, thus, arise either from the low activity of all of the molecules present or could reflect a low percentage of active molecules. To distinguish between these two possibilities, the percentage of active molecules was determined for each variant and wild-type factor. Previous studies have shown that both *E. coli* and mitochondrial EF-Tu display a lower percentage of active molecules in ternary complex formation than in GDP binding [23,27]. Thus, the percentage of molecules capable of forming a ternary complex with *E. coli* Phe-tRNA^{Phe} was determined for each factor.

As observed previously, the percentage of wild-type *E. coli* EF-Tu active in ternary complex formation is 25–35% [27]. However, the S221P variant has only approximately one-fifth of the active molecules as the wild-type factor. Thus, only about 5% of the total S221P variant is active in ternary complex formation (Fig. 3C). In agreement with previous data, the percentage of active wild-type EF-Tu$_{mt}$ molecules varies between 30% and 45% [23]. In comparison, EF-Tu$_{mt}$ P269S has 60–70% of the active molecules observed with the wild-type factor (Fig. 3C). This observation suggests that the mitochondrial variant is able to accommodate the proline to serine change with minimal loss in activity.

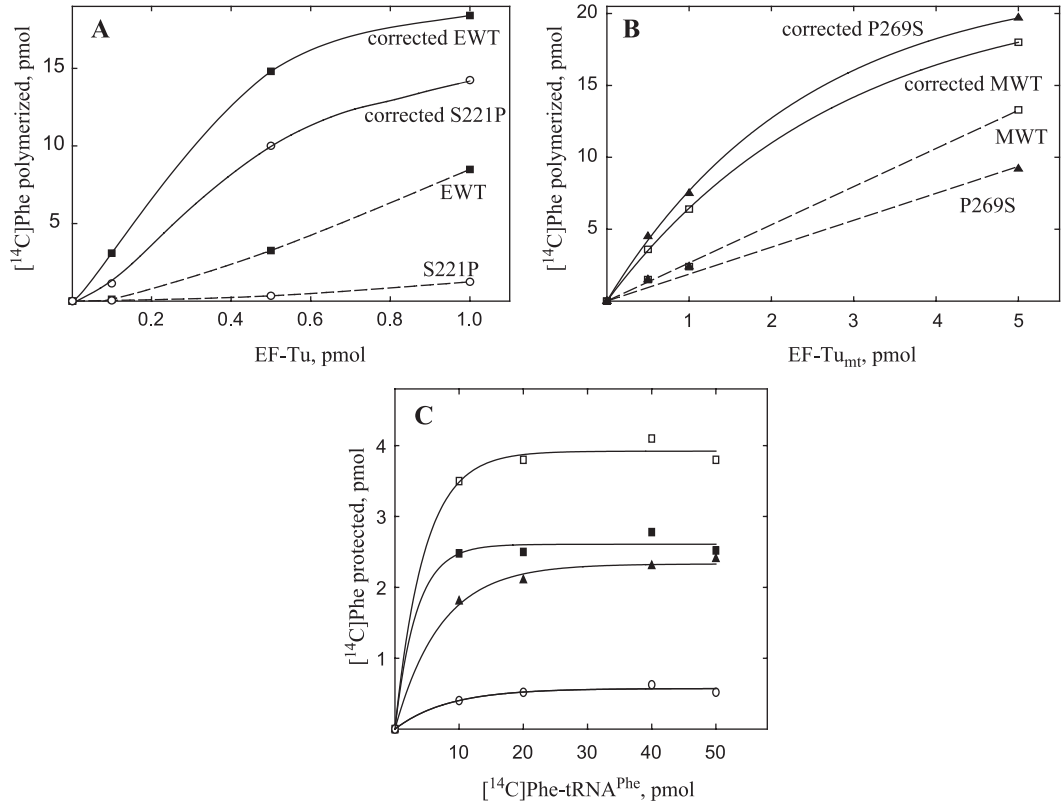

Fig. 3. Activity of *E. coli* EF-Tu and its S221P variant and determination of the percentage of active molecules: (A) Poly(U)-directed polymerization of [^{14}C]Phe was used to assay the activity of wild-type *E. coli* EF-Tu (EWT) (■) and *E. coli* EF-Tu S221P (○) before and after determining the percentage of molecules active in ternary complex formation. Dashed lines represent results obtained using the apparent pmol of EF-Tu as determined by the Bradford assay. Solid lines represent results using pmol of EF-Tu corrected for the percentage of molecules active in ternary complex formation. (B) Wild-type EF-Tu$_{mt}$ (MWT, □) and EF-Tu$_{mt}$ P269S (▲) in the absence of EF-Ts. Dashed lines represent results obtained using the apparent pmol of EF-Tu as determined by the Bradford assay. Solid lines represent results using pmol of EF-Tu corrected for the percentage of molecules active in ternary complex formation. (C) The percentage of molecules active in ternary complex formation for both *E. coli* EF-Tu S221P (○) and EF-Tu$_{mt}$ P269S (▲) are compared to those of wild-type *E. coli* EF-Tu (■) and wild-type EF-Tu$_{mt}$ (□), respectively. The ability of 0.2 μM EF-Tu (as determined by Bradford assays) to protect varying amounts of *E. coli* [^{14}C]Phe-tRNA^{Phe} from hydrolysis by RNase A was determined for each factor. The percentage of molecules active in ternary complex formation was determined from the point at which the factor was saturated with aa-tRNA.

After correcting the concentration of EF-Tu used in the in vitro translation assays for the percentage of active molecules, both *E. coli* EF-Tu S221P (Fig. 3A) and EF-Tu_{mt} P269S (Fig. 3B) display activities similar to their corresponding wild-type factors. These observations indicate that, although the S221P variant of *E. coli* EF-Tu has a low percentage of active molecules, those that are active function as well as the wild-type factor in poly(U)-directed synthesis. Thus, there is a small population of *E. coli* EF-Tu S221P molecules that are able to fully accommodate the serine to proline mutation and adopt the proper fold and function of the wild-type factor.

To further explore the apparently low percentage of active molecules in *E. coli* EF-Tu S221P, the percentage of molecules active in binding GDP was also determined and compared to that of wild-type *E. coli* EF-Tu (data not shown). In GDP binding, between 74% and 83% of the wild-type *E. coli* EF-Tu molecules are active as expected [27]. Similar to values determined with the ternary complex formation assays, *E. coli* EF-Tu S221P has only approximately one-seventh of the percentage of active molecules as the wild-type factor in GDP binding. Thus, the low percentage of active molecules in the *E. coli* EF-Tu S221P observed in ternary complex formation is not due to an alteration in the ability of the factor to bind a particular substrate but is indicative of the overall function of the variant. The decreased yield of *E. coli* EF-Tu S221P combined with the high percentage of inactive molecules compared to wild-type *E. coli* EF-Tu suggest that the factor has difficulty accommodating the replacement of Ser221 with Pro and that most of the molecules fail to fold into an active conformation.

The low recovery of the S221P variant of *E. coli* EF-Tu increases the possibility that the activity detected arises from contamination by the host factor lacking the His-tag. To test this possibility, *E. coli* BL21(DE3) cells carrying a plasmid coding for a variant of mitochondrial initiation factor 2 with an expression profile similar to that of *E. coli* EF-Tu S221P was expressed and purified via affinity chromatography alongside wild-type and variant *E. coli* EF-Tu. The activities of these preparations were then assayed in poly(U)-directed polymerization (data not shown). The activity detected in the preparations of the IF-2 variant was negligible compared to that of both wild-type *E. coli* EF-Tu and *E. coli* EF-Tu S221P, indicating that the activity observed in *E. coli* EF-Tu S221P is not due to the contaminating host factor.

3.4. Interaction of EF-Tu with E. coli aa-tRNA

Previous studies have shown that the active forms of both *E. coli* EF-Tu and EF-Tu_{mt} are able to bind *E. coli* Phe-$tRNA^{Phe}$ in the presence of GTP, forming stable ternary complexes [23]. As indicated in Fig. 3C, the S221P variant of *E. coli* EF-Tu and the P269S variant of EF-Tu_{mt} can form ternary complexes with Phe-$tRNA^{Phe}$. These experiments were carried out at a high concentration of Phe-$tRNA^{Phe}$ to determine the percentage of EF-Tu that was capable of binding the aa-tRNA. To obtain a better estimate of the relative abilities of *E. coli* EF-Tu S221P or EF-Tu_{mt} P269S to form ternary complexes, these variants were tested for their abilities to form ternary complexes in the presence of a limiting amount of *E. coli* Phe-$tRNA^{Phe}$. As indicated in Table 1, *E. coli* EF-Tu S221P and EF-Tu_{mt} P269S are capable of binding *E. coli* Phe-$tRNA^{Phe}$ as well as their respective wild-type factors. This observation indicates that any possible structural changes in the aa-tRNA binding pocket imposed by the mutations do not affect the ability of the correctly folded molecules to bind *E. coli* Phe-$tRNA^{Phe}$.

In the ternary complex involving Phe-$tRNA^{Phe}$ residue 221 is involved in creating the binding pocket for the 3′ end of the aa-tRNA, but it is not quite within hydrogen bond distance of C75 [8]. However, in the crystal structure of *T. aquaticus* EF-Tu complexed with GTP and *E. coli* Cys-$tRNA^{Cys}$, this residue (Thr232) directly forms a hydrogen bond with C75 of Cys-$tRNA^{Cys}$ [9]. Previous studies [28,29] have indicated that EF-Tu has different affinities for individual amino acids and tRNAs. In general, a strongly binding amino acid is coupled to a tRNA that binds EF-Tu less tightly. This thermodynamic compensation leads to similar affinities of all aa-tRNAs for EF-Tu. EF-Tu has a greater affinity for Phe than for Cys [28]. As a result, one expects this factor to have a higher affinity for $tRNA^{Cys}$ than for $tRNA^{Phe}$. The hydrogen bond observed between C75 and Ser221 in the ternary complex with Cys-$tRNA^{Cys}$ might

Table 1
Relative activities of normal and mutated forms of *E. coli* and mitochondrial EF-Tu

Activity tested	Relative activity			
	E. coli EF-Tu		EF-Tu_{mt}	
	Wild-type	S221P	Wild-type	P269S
3° complex with *E. coli* Phe-tRNA[a]	0.39	0.39	0.88	0.92
3° complex with *E. coli* Cys-tRNA[a]	0.28	0.32	0.26	0.35
3° complex with mitochondrial Phe-tRNA[a]	0.10	0.07	0.15	0.17
A-site binding with mitochondrial Phe-tRNA[b]	0.26	0.23	0.54	0.51
Polymerization with mitochondrial Phe-tRNA[c]	0.29	0.23	1.0	1.5

[a] Reactions were carried out at limiting concentrations of Phe-tRNA and EF-Tu as described in Materials and methods. The numbers reflect the pmol of 3° complex formed per pmol of EF-Tu. The values for *E. coli* EF-Tu are somewhat lower than those for EF-Tu_{mt} due to the sensitivity of *E. coli* EF-Tu to inhibition by GDP present in the preparation buffers.

[b] The values reported were obtained at 0.03 μM mitochondrial Phe-tRNA.

[c] Values reported were obtained at 0.01 μM EF-Tu and 0.1 μM mitochondrial Phe-tRNA.

play a role in enhancing the affinity of EF-Tu for this tRNA. The S221P mutation in *E. coli* EF-Tu disrupts this hydrogen bond and might, therefore, cause a decrease in the activity of *E. coli* EF-Tu S221P in ternary complex formation with Cys-tRNA^{Cys}. Likewise, one might expect an increase in activity for the P269S variant of EF-Tu$_{mt}$ since the serine residue should be able to form a stabilizing hydrogen bond with Cys-tRNA^{Cys}.

Ternary complex formation was, therefore, assayed for the wild-type and variant proteins using *E. coli* Cys-tRNA^{Cys}. Surprisingly, as indicated in Table 1, there is no significant decrease in the ability of the *E. coli* S221P variant to bind Cys-tRNA^{Cys} compared to wild-type *E. coli* EF-Tu. Thus, the hydrogen bond between residue 221 of *E. coli* EF-Tu and C75 of *E. coli* Cys-tRNA^{Cys} does not significantly contribute to the stability of the ternary complex with this aa-tRNA. Likewise, there is no increase in the ability of EF-Tu$_{mt}$ P269S to bind Cys-tRNA^{Cys}, again suggesting that this potential hydrogen bond does not play a significant role in aa-tRNA binding.

3.5. Interaction of EF-Tu with mitochondrial aa-tRNA

Mitochondrial aa-tRNAs lack many of the residues that form tertiary interactions in canonical tRNAs, and are thus thought to be less stable [3,4]. Previous studies have shown that *E. coli* EF-Tu is able to form ternary complexes with mitochondrial aa-tRNAs, but is unable to deliver them efficiently to the A-site of the ribosome [7]. We carried out ternary complex formation and A-site binding assays to determine the ability of each variant to bind to and deliver mitochondrial aa-tRNAs to the ribosome.

3.5.1. Ternary complex formation

The ability of EF-Tu to protect mitochondrial Phe-tRNA^{Phe} from hydrolysis by RNase A was analyzed for each wild-type and variant factor (Table 1). *E. coli* EF-Tu S221P is as active as wild-type *E. coli* EF-Tu in binding mitochondrial Phe-tRNA^{Phe}. Likewise, EF-Tu$_{mt}$ P269S exhibits a similar activity as that of wild-type EF-Tu$_{mt}$ in ternary complex formation. Overall, the ternary complex formation assays with the mitochondrial aa-tRNA suggest that this residue is not vital in the binding of mitochondrial Phe-tRNA^{Phe} to EF-Tu.

3.5.2. A-site binding to E. coli ribosomes

Studies monitoring the abilities of *E. coli* EF-Tu S221P and EF-Tu$_{mt}$ P269S to deliver *E. coli* Phe-tRNA^{Phe} to the A-site of *E. coli* ribosomes show that there is no significant difference in the activities of the variant factors and their respective wild-type counterparts (data not shown). In addition, under conditions in which EF-Tu is the only limiting reagent, all of the factors active in ternary complex formation are able to bind to and deliver *E. coli* Phe-tRNA^{Phe} to the A-site of poly(U)-programmed *E. coli* ribosomes (data not shown).

Additional experiments were carried out to examine the binding of mitochondrial Phe-tRNA^{Phe} to the A-site of the ribosome by the wild-type and variant factors. In A-site binding assays with the prokaryotic factors, *E. coli* EF-Tu S221P exhibits a similar activity as wild-type *E. coli* EF-Tu using mitochondrial Phe-tRNA^{Phe} and *E. coli* ribosomes (Table 1). It should be noted that *E. coli* EF-Tu has a limited ability to deliver mitochondrial Phe-tRNA^{Phe} to the A-site of the ribosome and is not as active as EF-Tu$_{mt}$ in this assay [7]. This difficulty probably arises from the lack of the canonical tertiary structure in these tRNAs. EF-Tu$_{mt}$ P269S also functions as well as wild-type EF-Tu$_{mt}$ in A-site binding with mitochondrial Phe-tRNA^{Phe} and *E. coli* ribosomes (Table 1).

A-site binding assays were also carried out with *E. coli* Phe-tRNA^{Phe} and mitochondrial ribosomes (data not shown). Again, the mutant and wild-type factors had similar activities. Therefore, the predicted conformational changes induced by the mutations do not interfere with or significantly promote the interaction between *E. coli* or mitochondrial EF-Tu with mitochondrial aa-tRNA and ribosomes.

3.5.3. Polymerization activity on mitochondrial ribosomes

The activities of the variants in polymerization with mitochondrial ribosomes and mitochondrial Phe-tRNA^{Phe} were then tested (Table 1). *E. coli* EF-Tu is significantly less active in assays using mitochondrial ribosomes and mitochondrial Phe-tRNA^{Phe} compared to EF-Tu$_{mt}$. Investigations of wild-type and variant *E. coli* EF-Tu show that the *E. coli* EF-Tu mutation of Ser221 to Pro does not improve the ability of the factor to function in polymerization with mitochondrial substrates.

Wild-type and P269S EF-Tu$_{mt}$ were also assayed for their abilities to carry out poly(U)-directed polymerization using mitochondrial Phe-tRNA^{Phe} and mitochondrial ribosomes. In such assays, EF-Tu$_{mt}$ P269S has an activity similar to that of the wild-type factor (Table 1). Thus, despite the observation that the Ser to Pro change between prokaryotic EF-Tu and EF-Tu$_{mt}$ occurs in a highly conserved region involved in aa-tRNA binding to Domain II, this position does not appear to be important in allowing EF-Tu$_{mt}$ to function in the organellar translational system.

4. Discussion

The major observation arising from the mutagenesis of Ser221 to Pro in *E. coli* EF-Tu is that the variant protein can only be expressed in low levels and that only a small percentage of the molecules present are actually folded into an active conformation. This observation stresses the importance of evaluating the percentage of active molecules in any assessment of the effect of a mutation on the activity of a protein. Clearly, *E. coli* EF-Tu poorly accommodates the structural rearrangements caused by the sub-

stitution of the Pro for a Ser residue at position 221. The five-member ring of proline imposes steric hindrance on the N-terminal side of the peptide bond and therefore restricts the range of motion of the bond. This added constraint may create a significant variation in the orientation of the loop in *E. coli* EF-Tu, resulting in a spatial arrangement resembling that in EF-Tu_{mt}. In addition, the structural alterations induced by the mutation are predicted to affect the solvent accessibility of proximal residues (Fig. 2B), exposing residues that are otherwise buried in *E. coli* EF-Tu and potentially significantly affecting the folded structure. Overall, much of the *E. coli* EF-Tu S221P variant fails to fold into a physiologically active form.

The structural rearrangements caused by the Ser to Pro substitution may also have affected the interdomain contacts between Domain I and Domains II/III. A significant difference between the mitochondrial and prokaryotic factors is observed in the spatial arrangement of Domain I relative to Domains II and III [3]. Domain I of EF-Tu_{mt} is rotated 12° relative to that of *E. coli* EF-Tu [3]. The S221P mutation in *E. coli* EF-Tu may have resulted in structural rearrangements resulting in interdomain contacts mimicking those of EF-Tu_{mt} and, thus inactivating much of the protein.

Conversely, it is evident that EF-Tu_{mt} P269S is more accommodating of the mutation-induced conformational changes, as the reduction in the percentage of active molecules compared to wild-type EF-Tu_{mt} is significantly less than that of *E. coli* EF-Tu S221P.

Perhaps the most surprising result arising from this work on *E. coli* EF-Tu is the lack of effect the mutation has on ternary complex formation with either *E. coli* or mitochondrial aa-tRNAs or on their delivery to the ribosome. The involvement of the loop containing this residue in binding the 3′ end of the aa-tRNA and the presence of a hydrogen bond between C75 of Cys-$tRNA^{Cys}$ and residue 221 suggest that this residue is of potential importance in the function of this factor. However, it is apparent from data reported here that Ser221 does not play a significant role in stabilizing the ternary complex or on its delivery to the ribosome.

It is intriguing that this Ser/Thr in the prokaryotic factors is replaced by Pro in the mammalian mitochondrial factors. The significant change in the nature of the amino acid present in this region of the aa-tRNA binding site suggests that this residue should be important in EF-Tu_{mt}. Structural models (Fig. 2A,B) indicate that the presence Pro269 significantly rearranges the binding pocket of the aa-tRNA (considering that the backbone of Domain II remains unchanged in the GDP, GDPNP and ternary complex structures of *T. aquaticus* EF-Tu). However, our results indicate that the Pro residue is not essential in the binding of mitochondrial Phe-$tRNA^{Phe}$ nor does it aid in the delivery of this aa-tRNA to the A-site of the ribosome.

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