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Interaction of Mitochondrial Elongation Factor Tu with Aminoacyl-tRNA and Elongation Factor Ts*

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Elongation factor (EF) Tu promotes the binding of aminoacyl-tRNA (aa-tRNA) to the acceptor site of the ribosome. This process requires the formation of a ternary complex (EF-Tu·GTP·aa-tRNA). EF-Tu is released from the ribosome as an EF-Tu·GDP complex. Exchange of GDP for GTP is carried out through the formation of a complex with EF-Ts (EF-Tu·Ts). Mammalian mitochondrial EF-Tu (EF-Tu_{mt}) differs from the corresponding prokaryotic factors in having a much lower affinity for guanine nucleotides. To further understand the EF-Tu_{mt} subcycle, the dissociation constants for the release of aa-tRNA from the ternary complex (K_{tRNA}) and for the dissociation of the EF-Tu·Ts_{mt} complex (K_{Ts}) were investigated. The equilibrium dissociation constant for the ternary complex was 18 ± 4 nM, which is close to that observed in the prokaryotic system. The kinetic dissociation rate constant for the ternary complex was 7.3 × 10^{-4} s^{-1}, which is essentially equivalent to that observed for the ternary complex in *Escherichia coli*. The binding of EF-Tu_{mt} to EF-Ts_{mt} is mutually exclusive with the formation of the ternary complex. K_{Ts} was determined by quantifying the effects of increasing concentrations of EF-Ts_{mt} on the amount of ternary complex formed with EF-Tu_{mt}. The value obtained for K_{Ts} (5.5 ± 1.3 nM) is comparable to the value of K_{tRNA}.

The classical model of the elongation cycle for protein synthesis is based on studies with *Escherichia coli*. In this model, the active form of elongation factor (EF)[1] Tu (the EF-Tu·GTP complex) binds aa-tRNA, forming a ternary complex. This ternary complex promotes the binding of aa-tRNA to the A-site of the ribosome. Once locked into the A-site by cognate codon-anticodon interactions, the GTP in the ternary complex is hydrolyzed, and EF-Tu is released from the ribosome as an EF-Tu·GDP complex. This complex must be dissociated by EF-Ts through the formation of an intermediate EF-Tu·Ts complex. GTP then replaces EF-Ts, reforming the active form of EF-Tu.

The equilibrium dissociation constant governing the affinity of *E. coli* EF-Tu for GDP is 8 nM, compared with 0.3 μM for GTP (1). The dissociation of the EF-Tu·GDP complex is thought to be the rate-limiting step in the recycling of EF-Tu (1). EF-Ts promotes the exchange of the tightly bound GDP for GTP through the formation of an intermediate EF-Tu·Ts complex. Presumably one of the reasons for the requirement for EF-Ts is because of the 40-fold higher affinity of EF-Tu for GDP compared with GTP. *E. coli* EF-Tu and EF-Ts have a high affinity for each other; the dissociation constant for the *E. coli* EF-Tu·Ts complex (K_{Ts}) is 2 nM (2). However, under intracellular conditions in *E. coli,* the EF-Tu·Ts complex appears to have a transient existence. The EF-Tu·Ts complex is rapidly converted to the ternary complex by high intracellular concentrations of GTP and by the tight binding of aa-tRNA to the EF-Tu·GTP complex. The dissociation constant for the release of aa-tRNA from the ternary complex is ~1 nM in *E. coli* (3).

EF-Tu is a highly conserved protein. Mammalian mitochondrial EF-Tu (EF-Tu_{mt}) has significant sequence identity (~55–60%) to the corresponding prokaryotic factors. Furthermore, structural analysis of bovine EF-Tu_{mt} indicates that the mammalian mitochondrial factor folds into a three-dimensional structure similar to that observed with *E. coli* and *Thermus thermophilus* EF-Tu (4–7). The sequence of EF-Ts is less conserved than that of EF-Tu, and distinct schemes are observed for the interaction of EF-Tu and EF-Ts in different systems. In *T. thermophilus*, a heterotetrameric complex ((EF-Tu·$Ts)_2$) occurs through the interaction of two EF-Tu molecules with a stable EF-Ts dimer (8). Unlike the *E. coli* EF-Tu·Ts complex, the *T. thermophilus* complex is not dissociated to a significant extent by either GDP or GTP alone (9). No elongation factor equivalent to EF-Ts has been identified in yeast mitochondria or in the complete sequence of the yeast genome, perhaps reflecting the observation that yeast EF-Tu_{mt} has a very low affinity for guanine nucleotides (10).

Unlike the *E. coli* EF-Tu·Ts complex, the bovine liver EF-Tu·Ts_{mt} complex cannot be dissociated in the presence of high concentrations of guanine nucleotides (11). This observation is due, at least in part, to a low affinity of EF-Tu_{mt} for GDP and GTP. The equilibrium dissociation constants for the EF-Tu_{mt}·GDP and EF-Tu_{mt}·GTP complexes are ~2 orders of magnitude higher than those observed for *E. coli* EF-Tu.[2] However, in the presence of GTP and aa-tRNA, the EF-Tu·Ts_{mt} complex dissociates, and a classical ternary complex is observed (13, 14). Thus, the basic steps of the bacterial elongation cycle appear to occur in mammalian mitochondria. However, the equilibrium constants that govern the interaction of EF-Tu_{mt} with EF-Ts_{mt} and with guanine nucleotides appear to be significantly different. To facilitate the understanding of the translational elongation cycle in mammalian mitochondria, the equilibrium dis-

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[1] The abbreviations used are: EF, elongation factor; aa-tRNA, aminoacyl-tRNA; mt, mitochondrial; mantGDP, 2′(or 3′)-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate.

[2] Y.-C. Cai, J. M. Bullard, N. L. Thompson, and L. L. Spremulli, submitted for publication.

sociation constants governing the release of aa-tRNA from the ternary complex and the dissociation of the EF-Tu·Ts_{mt} complex are reported here.

MATERIALS AND METHODS

Buffers—Buffer I was composed of 8 mM Hepes-KOH, pH 7.6, 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 76 mM KCl, 6.8 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 3.4 units of pyruvate kinase, and 0.5 mM GTP. Buffer II was composed of 20 mM Hepes-KOH, pH 7.0, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol.

Expression and Purification of EF-Tu_{mt} and EF-Ts_{mt}—EF-Tu_{mt} and EF-Ts_{mt} expressed in *E. coli* as His-tagged proteins were purified as described previously (15). EF-Ts_{mt} was further purified by incubating the sample (prepared from 6 liters of cell culture) with 150 μl of DEAE-cellulose resin for 15 min at 4 °C. The DEAE-cellulose resin was removed by centrifugation in an Eppendorf tube at 14,000 rpm for 5 min, and EF-Ts_{mt} remained in the supernatant. To determine an accurate protein concentration, ~950 pmol of EF-Ts_{mt} (determined by the micro-Bradford method) in buffer II (90 μl) were denatured in 6 M guanidinium chloride (total of 300 μl) by incubation at 50 °C for at least 5 h (16). The concentration of EF-Ts_{mt} was then calculated from the absorbance of the sample at 280 nm using an extinction coefficient of 38,370 M^{-1} cm^{-1}, calculated by the procedure of Gill and von Hippel (17).

Measurement of Ternary Complex Formation—Ternary complex formation was monitored by taking advantage of the ability of EF-Tu_{mt} to protect [^{14}C]Phe-tRNA^{Phe} from digestion by RNase A. Reaction mixtures were incubated with the indicated amounts of EF-Tu_{mt} and [^{14}C]Phe-tRNA in buffer I on ice for 20 min. The GDP present in the EF-Tu_{mt} preparations was converted to GTP by phosphoenolpyruvate and pyruvate kinase during this incubation. The reaction was stopped by treating with RNase A (40 μg/ml), incubating for 30 s on ice, and adding 3 ml of ice-cold 5% trichloroacetic acid. The precipitate was collected after 10 min on ice by filtering through nitrocellulose membrane filters to test for the amount of [^{14}C]Phe-tRNA remaining (18, 19). The RNase A concentration was optimized by dosing RNase A in reactions containing 10 pmol of [^{14}C]Phe-tRNA.

The percentage of EF-Tu_{mt} active in forming ternary complexes was determined by increasing the concentration of [^{14}C]Phe-tRNA to saturating levels (1 μM) in 50-μl reaction volumes while keeping the concentration of EF-Tu_{mt} at 0.2 μM and the concentration of GTP at 0.5 mM. A blank representing the amount of [^{14}C]Phe-tRNA remaining after nuclease digestion was done at each [^{14}C]Phe-tRNA concentration. This blank (<0.5 pmol) was used to correct for variations in RNase activity at different concentrations of [^{14}C]Phe-tRNA. The maximal concentration of ternary complex detected was assumed to be equivalent to the concentration of EF-Tu_{mt} capable of forming ternary complexes.

The equilibrium dissociation constant governing the release of [^{14}C]Phe-tRNA from the ternary complex was determined using a range of EF-Tu_{mt} (0.02–1 μM) and [^{14}C]Phe-tRNA (0.04–0.09 μM) concentrations. The concentrations given reflect only the active EF-Tu_{mt} in each preparation. The effects of spermine (0.5–2.0 μM) and spermidine (16–64 μM) on ternary complex formation were examined by incubating these reagents with EF-Tu (0.4 μM) and [^{14}C]Phe-tRNA (~0.06, 0.1, and 0.15 μM) as described above.

The kinetic dissociation rate for the ternary complex EF-Tu_{mt}·GTP·Phe-tRNA was measured as described (3). In this reaction, 0.015 μM [^{14}C]Phe-tRNA and 0.015 μM EF-Tu_{mt} were incubated with 10, 100, and 500 μM GTP separately in 6.5 ml of buffer I as described above. After incubation on ice for 15 min, RNase A (40 μg/ml) was added. The dissociation of [^{14}C]Phe-tRNA was monitored by removing aliquots (0.5 ml) at different times (30 s to 6 min). The amount of ternary complex remaining was determined as described above.

Measurement of the Equilibrium Dissociation Constant for the EF-Tu·Ts_{mt} Complex—The equilibrium dissociation constant governing the EF-Tu·Ts_{mt} complex was determined by competition with ternary complex formation. EF-Tu_{mt} (1 μM) was combined with different concentrations of EF-Ts_{mt} (0.2–6 μM) in buffer II (100 μl). Aliquots (10 μl) were diluted 5-fold with buffer I containing different concentrations of [^{14}C]Phe-tRNA (providing final concentrations of Phe-tRNA from 0.06 to 1.4 μM). An additional series of samples (in 50 μl of buffer I) was prepared containing 2.1 μM [^{14}C]Phe-tRNA, 0.1 μM EF-Tu_{mt}, and various concentrations of EF-Ts_{mt} (0.03–0.7 μM). All samples were incubated on ice for 20 min. RNase A (2 μg for <0.6 μM [^{14}C]Phe-tRNA and 5 μg for >0.6 μM [^{14}C]Phe-tRNA) was then added, and the samples were incubated for 30 s on ice. The nuclease reaction was stopped by the addition of 3 ml of cold 5% trichloroacetic acid, and the amount of [^{14}C]Phe-tRNA protected by EF-Tu_{mt} was measured as described above. The maximal concentration of ternary complex detected was assumed to be equivalent to the concentration of EF-Tu_{mt} in the EF-Tu·Ts_{mt} mixtures capable of forming ternary complexes.

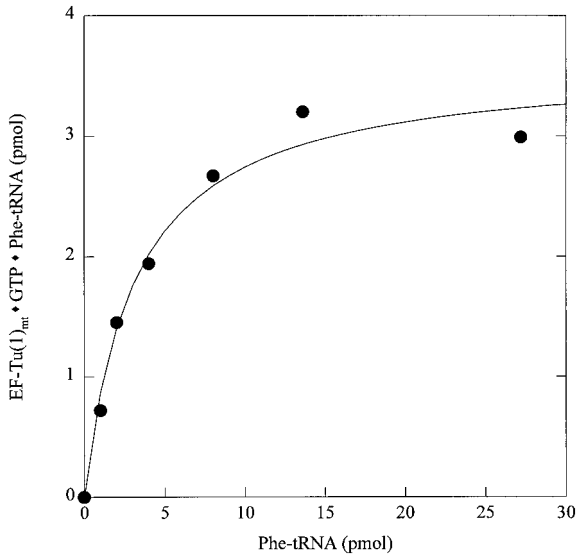

FIG. 1. **Percentage of EF-Tu_{mt} active in ternary complex formation.** EF-Tu_{mt} (10 pmol) was incubated with increasing concentrations of Phe-tRNA containing trace amounts of [^{14}C]Phe-tRNA. The amount of ternary complex formed was determined using the nuclease protection assay described under "Materials and Methods."

RESULTS

Equilibrium Dissociation Constant for the Release of aa-tRNA from the Ternary Complex—The function of EF-Tu is to guide aa-tRNA into the A-site of the ribosome. This step requires the formation of a ternary complex composed of EF-Tu, aa-tRNA, and GTP. Since EF-Tu_{mt} forms functional ternary complexes with aa-tRNAs from *E. coli*, we used *E. coli* Phe-tRNA^{Phe} to measure the equilibrium dissociation constant governing the ternary complex formed with EF-Tu_{mt} (13).

The amount of ternary complex present at different concentrations of EF-Tu_{mt} and Phe-tRNA was measured by a ribonuclease protection assay (18, 20). The time course of ternary complex formation indicated that the formation of these complexes was complete in <1 min under the conditions used (data not shown). However, we routinely used a 15-min incubation for ternary complex formation to ensure that the reactions reached equilibrium and to simplify processing.

Since aa-tRNA can deacylate over time by the hydrolysis of the ester bond between the amino acid and the tRNA, the percentage of the radiolabel present as [^{14}C]Phe-tRNA in the preparations was monitored using a trichloroacetic acid precipitation assay. In some preparations, as much as 30% of the [^{14}C]Phe-tRNA had undergone spontaneous hydrolysis to free [^{14}C]Phe and tRNA. Essentially all of the [^{14}C]Phe-tRNA remaining in each preparation could be protected from RNase digestion in the presence of excess EF-Tu_{mt}, indicating that essentially 100% of the [^{14}C]Phe-tRNA was active in ternary complex formation (data not shown). This value was determined in each experiment and used to calculate the concentration of Phe-tRNA present.

The percentage of EF-Tu_{mt} active in ternary complex formation was measured by dosing the amount of Phe-tRNA added to a constant concentration of EF-Tu_{mt} (Fig. 1). In the presence of excess Phe-tRNA, the maximal concentration of ternary complex detected is equivalent to the concentration of EF-Tu_{mt} capable of binding to Phe-tRNA. These assays indicated that ~30% of the EF-Tu_{mt} in most preparations was active in ternary complex formation. A similar observation has been made

for the native EF-Tu_{mt} obtained from bovine liver (18), indicating that this value is not the result of the use of recombinant EF-Tu_{mt}. In *E. coli*, ~80% of the EF-Tu is capable of binding GDP, whereas only 30–40% can form ternary complexes (3). The underlying reason for this difference is not known. In subsequent discussions, the EF-Tu_{mt} that is capable of binding aa-tRNA is denoted EF-$Tu(1)_{mt}$, and the EF-Tu_{mt} that is not capable of binding aa-tRNA is denoted EF-$Tu(2)_{mt}$.

In *E. coli*, GTP can bind free EF-Tu, whereas aa-tRNA cannot bind to EF-Tu in the absence of GTP (21). Previous work indicates that a similar sequence of events occurs in the mammalian mitochondrial system (14). Hence, in the analysis carried out here, EF-Tu_{mt} is assumed to bind to GTP first, followed by the binding of aa-tRNA. The formation of the ternary complex is then described by the following two coupled reactions (Equations 1 and 2).

$$\text{EF-Tu(1)}_{mt} + \text{GTP} \rightleftharpoons \text{EF-Tu(1)}_{mt}\cdot\text{GTP} \quad \text{(Eq. 1)}$$

$$\text{EF-Tu(1)}_{mt}\cdot\text{GTP} + \text{aa-tRNA} \rightleftharpoons \text{EF-Tu(1)}_{mt}\cdot\text{GTP}\cdot\text{aa-tRNA} \quad \text{(Eq. 2)}$$

The equilibrium dissociation constants controlling these two steps are as follows (Equations 3 and 4).

$$K_{GTP} = \frac{[\text{EF-Tu(1)}_{mt}][\text{GTP}]}{[\text{EF-Tu(1)}_{mt}\cdot\text{GTP}]} \quad \text{(Eq. 3)}$$

$$K_{tRNA} = \frac{[\text{EF-Tu(1)}_{mt}\cdot\text{GTP}][\text{aa-tRNA}]}{[\text{EF-Tu(1)}_{mt}\cdot\text{GTP}\cdot\text{aa-tRNA}]} \quad \text{(Eq. 4)}$$

The total concentration of aa-tRNA is given by Equation 5.

$$[\text{aa-tRNA}]_t = [\text{aa-tRNA}] + [\text{EF-Tu(1)}_{mt}\cdot\text{GTP}\cdot\text{aa-tRNA}] \quad \text{(Eq. 5)}$$

In the assays described here, the concentration of GTP was 0.5 mM, which was much greater than the concentrations of EF-Tu_{mt} (<0.4 μM) and aa-tRNA (<1 μM). At such high GTP concentrations, >96% of the EF-Tu_{mt} binds to GTP as determined by the value of K_{GTP} (18 μM) in the absence of aa-tRNA.[2] Therefore, it was assumed that no free EF-Tu_{mt} was present in these assays. Under these conditions, the total concentration of EF-$Tu(1)_{mt}$ is given by Equation 6.

$$[\text{EF-Tu(1)}_{mt}]_t = [\text{EF-Tu(1)}_{mt}\cdot\text{GTP}] + [\text{EF-Tu(1)}_{mt}\cdot\text{GTP}\cdot\text{aa-tRNA}] \quad \text{(Eq. 6)}$$

The dissociation constant governing the ternary complex was determined by measuring the concentration of the ternary complex, [EF-$Tu(1)_{mt}$·GTP·Phe-tRNA], formed at various total concentrations of EF-$Tu(1)_{mt}$ and Phe-tRNA (Fig. 2). [EF-$Tu(1)_{mt}$·GTP·Phe-tRNA] was determined by the RNase A protection assay. $[\text{EF-Tu(1)}_{mt}]_t$ was determined by the concentration of EF-Tu_{mt} active in forming the ternary complex, which was measured in each assay by the approach described above. These values then permitted calculation of [EF-$Tu(1)_{mt}$·GTP] (Equation 6). $[\text{Phe-tRNA}]_t$ was determined from the amount of ternary complex formed at saturating concentrations of EF-Tu_{mt} for each Phe-tRNA concentration or by the trichloroacetic acid precipitation method. Free [Phe-tRNA] was calculated from the values of $[\text{Phe-tRNA}]_t$ and [EF-$Tu(1)_{mt}$·GTP·Phe-tRNA] using Equation 5. K_{tRNA} was then calculated using Equation 4. The equilibrium dissociation constant for the ternary complex was determined to be 18 ± 4 nM based on 16 independent assays.

A representative data set showing the concentration of the ternary complex for a given constant value of $[\text{Phe-tRNA}]_t$ and as a function of $[\text{EF-Tu(1)}_{mt}]_t$ is shown in Fig. 2. Equations 4–6 imply the following (Equation 7).

$$2[EF\text{-}Tu(1)_{mt}\cdot GTP\cdot aa\text{-}tRNA] = [EF\text{-}Tu(1)_{mt}]_t + [aa\text{-}tRNA]_t + K_{tRNA} - \{([EF\text{-}Tu(1)_{mt}]_t + [aa\text{-}tRNA]_t + K_{tRNA})^2 - 4[EF\text{-}Tu(1)_{mt}]_t[aa\text{-}tRNA]_t\}^{1/2} \quad \text{(Eq. 7)}$$

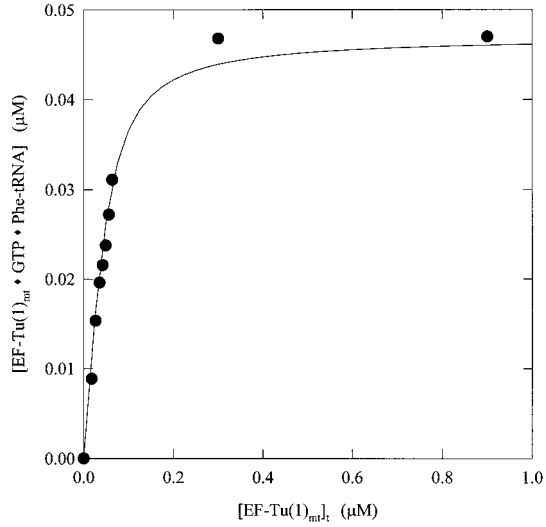

FIG. 2. **Formation of the ternary complex.** Different concentrations of EF-Tu_{mt} were incubated with 47 nM Phe-tRNA. The amount of Phe-tRNA protected by EF-Tu_{mt} is plotted as a function of the total EF-Tu_{mt} concentration. The *solid line* was obtained from Equation 7 with K_{tRNA} = 18 nM.

As shown in Fig. 2, the data agree well with the values of [EF-$Tu(1)_{mt}$·GTP·Phe-tRNA] as predicted by Equation 7 for the known values of $[\text{Phe-tRNA}]_t$, $[\text{EF-Tu(1)}_{mt}]_t$ and K_{tRNA} = 18 nM.

Polyamine Effects on the Formation of the Ternary Complex—The crystal structure of the ternary complex indicates that the backbone of the TψC stem of the aa-tRNA interacts with side chains of EF-Tu through a series of electrostatic contacts (22, 23). Several Lys residues have been reported to play an important role in the formation of the ternary complex (24). Consequently, ionic conditions are expected to influence the formation of the ternary complex.

Polyamines are known to play a role in a number of reactions involving tRNAs (25). To test the effect of polyamines on the formation of the ternary complex, different concentrations of spermine and spermidine were incubated with limiting concentrations of EF-Tu_{mt} and [^{14}C]Phe-tRNA. Spermine inhibited the formation of the ternary complex even at extremely low concentrations (Fig. 3*A*). Spermidine also had an inhibitory effect on ternary complex formation (Fig. 3*B*), although higher concentrations (>10-fold) were needed to have the same inhibitory effect as spermine. This difference may arise from the fact that spermidine has three positive charges, whereas spermine can have four positive charges. Polyamines probably interact with the backbone of the tRNA, masking some of its negative charge and preventing interaction with EF-Tu_{mt}.

Dissociation Rate Constant for the Ternary Complex—The rate of dissociation of the ternary complex was analyzed using a modification of the RNase protection assay (3). For these experiments, the ternary complex was allowed to form. Subsequently, RNase A was added to the reaction mixture. As the Phe-tRNA dissociated from the complex, it was rapidly hydrolyzed by the RNase present. This step prevented the reassociation of the Phe-tRNA with EF-Tu_{mt} and allowed the quantitation of the amount of ternary complex remaining. Samples were removed at different times after the addition of the RNase, and the ternary complex remaining was measured by cold trichloroacetic acid precipitation.

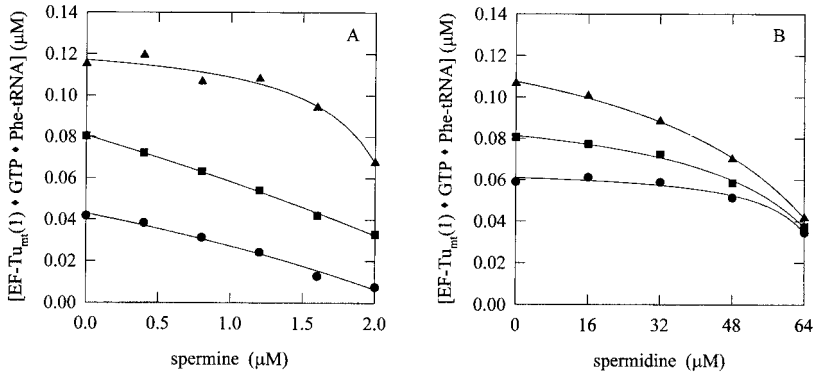

FIG. 3. **Effects of polyamines on the formation of the ternary complex.** Different concentrations of spermine (*A*; 0.5–2.0 μM) or spermidine (*B*; 16–64 μM) were incubated with EF-Tu$_{mt}$ (0.4 μM) and one of three concentrations of Phe-tRNA in buffer I (50 μl) on ice for 20 min. In *A*, the Phe-tRNA concentrations were 0.06 μM (●), 0.10 μM (■), and 0.15 μM (▲). In *B*, the Phe-tRNA concentrations were 0.07 μM (●), 0.09 μM (■), and 0.16 μM (▲). Ternary complex formation was determined by the ribonuclease protection assay.

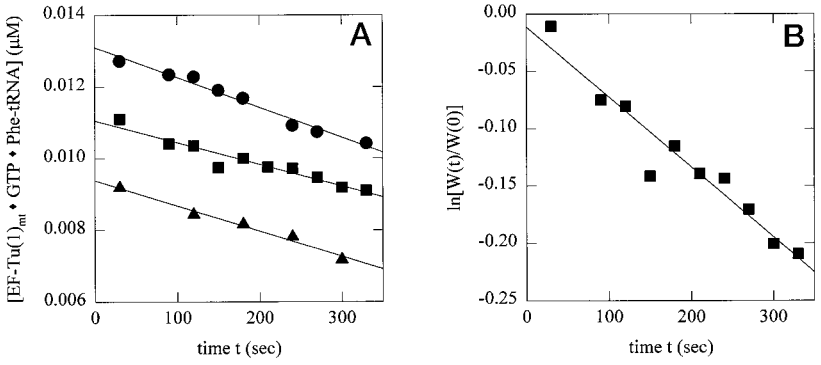

FIG. 4. **Dissociation rate for the ternary complex.** Experimental data for the determination of the dissociation rate for complexes formed between EF-Tu$_{mt}$·GTP and Phe-tRNA are shown. Each ternary complex was equilibrated in 6.5 ml of reaction buffer in the presence of EF-Tu$_{mt}$ (0.015 μM) and Phe-tRNA (0.015 μM). The concentration of GTP was 10 μM (●), 100 μM (■), or 500 μM (▲). After 15 min on ice, 40 μg/ml RNase A was added. Aliquots of 0.5 or 1 ml were removed after 0.5–5.5 min and processed as described under "Materials and Methods." *A*, the ternary complex concentration decreases as a function of time. *B*, shown is a representative plot of the time dependence of the natural logarithm of W(t)/W(0), where W(t) is the time-dependent concentration of the ternary complex. The data are linear, indicating first-order dissociation kinetics (Equation 8). Data like these were fit by linear least-squares analysis to find the value of the dissociation rate constant k.

The dissociation of the ternary complex follows first-order kinetics and is described by Equation 8,

$$W(t) = W(0)e^{-kt} \quad \text{(Eq. 8)}$$

where W(t) is the concentration of the ternary complex at time t, W(0) is the initial concentration of the ternary complex, and k is the dissociation rate constant. The natural logarithm of W(t)/W(0) was plotted as a function of time, and the data were analyzed by least-squares linear fits. The dissociation rate constant for the ternary complex was determined from the slope. Three different concentrations of GTP were used (Fig. 4). The rate constant was very similar at each concentration since the GTP concentrations were much higher than the EF-Tu$_{mt}$ concentrations (at least 600-fold higher). The dissociation rate constant was determined to be $(7.3 \pm 1.3) \times 10^{-4}$ s^{-1}. The dissociation rate constant for the ternary complex formed with *E. coli* EF-Tu and *E. coli* Phe-tRNA^{Phe} is very similar to that observed with EF-Tu$_{mt}$ (17×10^{-4} s^{-1}) (3).

Equilibrium Dissociation Constant for the EF-Tu·Ts$_{mt}$ Complex—In previous work, the equilibrium dissociation constant for EF-Tu$_{mt}$ and a fluorescent analog of GDP, mantGDP, was found to be ~2 μM.[2] Initially, competition experiments between mantGDP and EF-Ts$_{mt}$ for binding to EF-Tu$_{mt}$ were undertaken in an effort to obtain an estimate of the equilibrium dissociation constant for the EF-Tu·Ts$_{mt}$ complex (K_{Ts}). However, the affinity of EF-Ts$_{mt}$ for EF-Tu$_{mt}$ was too high compared with the affinity of mantGDP for EF-Tu$_{mt}$ to allow an accurate determination of K_{Ts} by this method. Therefore, competition between EF-Ts$_{mt}$ and GTP plus Phe-tRNA for binding to EF-Tu$_{mt}$ was developed as an approach to determine K_{Ts}.

The percentage of EF-Ts$_{mt}$ molecules active in binding EF-Tu$_{mt}$ was shown to be close to 100% using gel filtration chromatography and several other methods (data not shown). EF-Tu$_{mt}$ was also determined to be fully active in forming the EF-Tu·Ts$_{mt}$ complex using saturating amounts of biotinylated EF-Ts$_{mt}$ and avidin-conjugated beads (data not shown).

As described above, free EF-Tu$_{mt}$ was generally ~30% active in forming the ternary complex. However, in the presence of EF-Ts$_{mt}$, the percentage of EF-Tu$_{mt}$ active in ternary complex formation increased. Therefore, it was essential to obtain an estimate of the percentage of EF-Tu$_{mt}$ active in ternary complex formation as a function of the concentration of EF-Ts$_{mt}$. To determine these values, EF-Tu$_{mt}$ was incubated with different amounts of EF-Ts$_{mt}$ and then tested for the ability to form ternary complexes at different concentrations of Phe-tRNA. At higher concentrations of Phe-tRNA and saturating concentrations of GTP, EF-Tu$_{mt}$ will form the ternary complex instead of forming the EF-Tu·Ts$_{mt}$ complex. The maximal amount of the ternary complex that can be formed reflects the percentage of EF-Tu$_{mt}$ active in binding Phe-tRNA in each sample.

Plotting the percentage of active EF-Tu$_{mt}$ obtained as described above as a function of the amount of EF-Ts$_{mt}$ added indicated that, in the presence of EF-Ts$_{mt}$, the percentage of EF-Tu$_{mt}$ active in forming the ternary complex increased from 26 to 41% (Fig. 5). This phenomenon was also observed with *E. coli* EF-Tu in the presence of *E. coli* EF-Ts (data not shown). This activation of EF-Tu$_{mt}$ increased until the ratio of EF-Tu$_{mt}$ to EF-Ts$_{mt}$ was ~1:1.

Representative data for the competition between EF-Ts$_{mt}$ and Phe-tRNA for binding EF-Tu$_{mt}$ are shown in Fig. 6. The amount of ternary complex formed clearly decreases as the concentration of EF-Ts$_{mt}$ is increased. The analysis of these

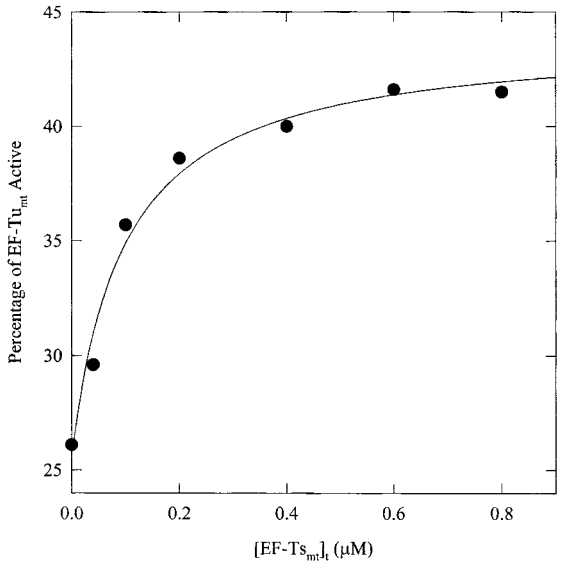

FIG. 5. **Effect of the amount of EF-Ts$_{mt}$ on the percentage of EF-Tu$_{mt}$ active in forming the ternary complex.** EF-Tu$_{mt}$ (0.2 μM) was incubated with various concentrations of EF-Ts$_{mt}$. These mixtures were tested for the percentage of EF-Tu$_{mt}$ active in forming the ternary complex. For this determination, each mixture of EF-Tu$_{mt}$ and EF-Ts$_{mt}$ was tested for the maximal amount of ternary complex that could be formed (see Fig. 1). The data here represent the plateau values for ternary complex formed at saturating levels of Phe-tRNA.

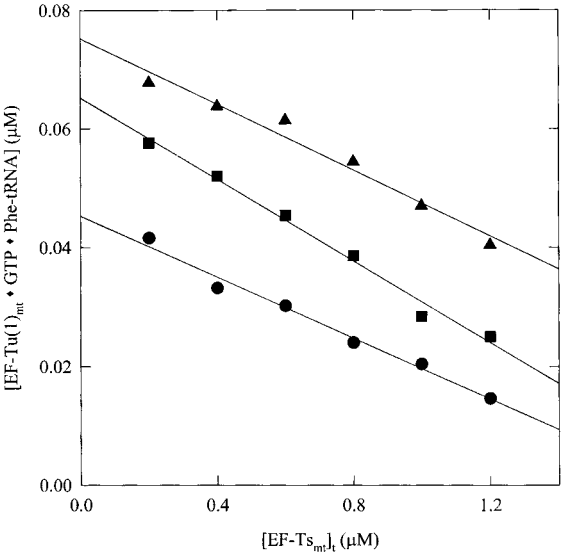

FIG. 6. **Competition of EF-Ts$_{mt}$ with GTP and [^{14}C]Phe-tRNA for binding to EF-Tu$_{mt}$.** Several concentrations of EF-Ts$_{mt}$ (0.2–1.2 μM) were used to compete with Phe-tRNA for EF-Tu$_{mt}$ (0.2 μM) in 50-μl reaction volumes as described under "Materials and Methods." The total concentration of Phe-tRNA was 0.065 μM (●), 0.14 μM (■), or 0.34 μM (▲). The amount of ternary complex formed was determined using the RNase protection assay and is plotted as a function of the total concentration of EF-Ts$_{mt}$. The *lines* represent linear least-squares fits.

data for the determination of K_{Ts} was as follows. Depending on the amount of EF-Ts$_{mt}$ present, 30–40% of the EF-Tu$_{mt}$ was active in forming the ternary complex. However, all of the EF-Tu$_{mt}$ was active in binding to EF-Ts$_{mt}$. This difference suggests that two forms of EF-Tu$_{mt}$ exist in the system at any given concentration of EF-Ts$_{mt}$. Form 1 of EF-Tu$_{mt}$, denoted EF-Tu(1)$_{mt}$, is active in binding EF-Ts$_{mt}$ and in forming the ternary complex. Form 2 of EF-Tu$_{mt}$, denoted EF-Tu(2)$_{mt}$, is active in forming the EF-Tu·Ts$_{mt}$ complex, but is not active in

TABLE I
Equilibrium dissociation constants for EF-Tu from bovine mitochondria and E. coli with guanine nucleotides, Phe-tRNA, and EF-Ts

EF-Tu	K_{GDP}	K_{GTP}	K_{tRNA}	K_{Ts}	Ref.
	μM	μM	nM	nM	
E. coli	0.0077	0.3	1.1	2	2, 3, 21
Bovine mitochondria	1.0 ± 0.4	18 ± 6	18 ± 4	5.5 ± 1.3	This work[a]

[a] Y.-C. Cai, J. M. Bullard, N. L. Thompson, and L. L. Spremulli, submitted for publication.

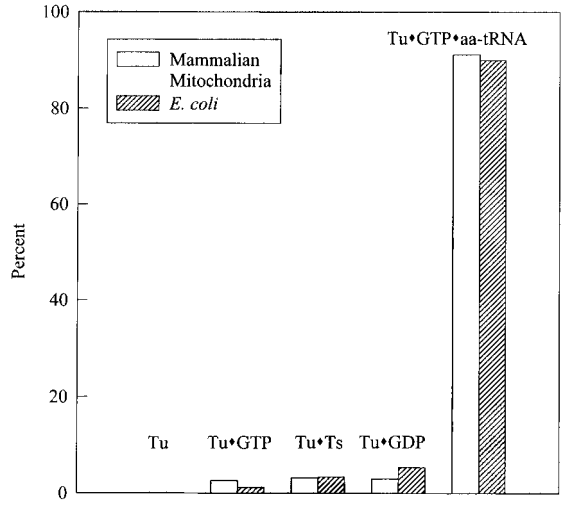

FIG. 7. **Composition of different forms of EF-Tu in *E. coli* and mammalian mitochondria.** The fraction of EF-Tu present in various complexes was calculated based on the data listed in Tables I and II.

forming the ternary complex. Previous studies have shown that EF-Tu$_{mt}$ is nearly 100% active in guanine nucleotide binding, indicating that EF-Tu(2)$_{mt}$ can bind GTP.[2]

The possible interactions are described by Equations 1 and 2 and the following three additional equilibria, where $n = 1$ or 2 (Equations 9 and 10).

$$\text{EF-Tu}(n)_{mt} + \text{EF-Ts}_{mt} \rightleftharpoons \text{EF-Tu}(n)\cdot\text{Ts}_{mt} \quad \text{(Eq. 9)}$$

$$\text{EF-Tu(2)}_{mt} + \text{GTP} \rightleftharpoons \text{EF-Tu(2)}_{mt}\cdot\text{GTP} \quad \text{(Eq. 10)}$$

The equilibrium dissociation constants are given by Equations 3, 4, 11, and 12.

$$K_{Ts} = \frac{[\text{EF-Tu}(n)_{mt}][\text{EF-Ts}_{mt}]}{[\text{EF-Tu}(n)\cdot\text{Ts}_{mt}]} \quad \text{(Eq. 11)}$$

$$K_{GTP} = \frac{[\text{EF-Tu(2)}_{mt}][\text{GTP}]}{[\text{EF-Tu(2)}_{mt}\cdot\text{GTP}]} \quad \text{(Eq. 12)}$$

In each assay, the total concentrations of Phe-tRNA and EF-Ts$_{mt}$ were known from the starting conditions and are given by Equations 5 and 13.

$$[\text{EF-Ts}_{mt}]_t = [\text{EF-Ts}_{mt}] + [\text{EF-Tu(1)}\cdot\text{Ts}_{mt}] + [\text{EF-Tu(2)}\cdot\text{Ts}_{mt}] \quad \text{(Eq. 13)}$$

The total concentrations of the two forms of EF-Tu$_{mt}$ were determined by the percentage activity for forming the ternary complex as described above and are given by Equations 14 and 15.

$$[\text{EF-Tu(1)}_{mt}]_t = [\text{EF-Tu(1)}_{mt}] + [\text{EF-Tu(1)}_{mt}\cdot\text{GTP}] + [\text{EF-Tu(1)}\cdot\text{Ts}_{mt}] + [\text{EF-Tu(1)}_{mt}\cdot\text{GTP}\cdot\text{aa-tRNA}] \quad \text{(Eq. 14)}$$

$$[\text{EF-Tu(2)}_{mt}]_t = [\text{EF-Tu(2)}_{mt}] + [\text{EF-Tu(2)}_{mt}\cdot\text{GTP}] + [\text{EF-Tu(2)}\cdot\text{Ts}_{mt}] \quad \text{(Eq. 15)}$$

TABLE II

Estimated amounts and concentrations of translational components in E. coli and bovine liver mitochondria

Values were estimated as the number of copies per mitochondrion or per *E. coli* cell.

Component	Mammalian mitochondria	*E. coli*[a]	Refs.
Ribosomes	~80–100 (0.06–0.075 μM)	~25,000 (0.02 mM)	27, 28
EF-Tu	~400 (0.3 μM)[b]	~200,000 (0.15 mM)	11, 29, 30
EF-Ts	~400 (0.3 μM)	~25,000 (0.02 mM)	1, 11
tRNA	~1200 (0.9 μM)	~180,000 (0.135 mM)	28, 31
GTP	(0.79 mM)	(1 mM)	12, 28, 32
GDP	(0.05 mM)	(0.1 mM)	12, 28, 32

[a] The concentration of translational components in *E. coli* varies considerably with the growth rate of the cell. The values used here are for an intermediate growth rate (a doubling time of ~40 min). An excellent description of the changes in the concentrations of the translational machinery can be found in Ref. 26. The values for mitochondria are taken from HeLa cells (doubling time of ~24 h) and from liver and heart (nongrowinng tissues). There is currently no clear indication that these values change significantly with growth rate.

[b] Calculated based on information contained in Ref. 11.

For given amounts of $[EF\text{-}Tu(1)_{mt}]_t$, $[EF\text{-}Tu(2)_{mt}]_t$, $[GTP]_t$, $[aa\text{-}tRNA]_t$, and $[EF\text{-}Ts_{mt}]_t$, the concentration of the ternary complex, denoted $[EF\text{-}Tu(1)_{mt}\cdot GTP\cdot aa\text{-}tRNA]$, was determined by the nuclease protection assay as described above (Fig. 6). The concentration of free Phe-tRNA was then calculated using Equation 5. Because the value of K_{tRNA} had been determined, the value of $[EF\text{-}Tu(1)_{mt}\cdot GTP]$ could then be calculated from Equation 4. The total concentration of GTP (0.50 mM) was much higher than the total concentration of $EF\text{-}Tu_{mt}$ (≤0.2 μM) or Phe-tRNA (≤2 μM). Therefore, it was assumed that the concentration of free GTP was equal to the total concentration of GTP. $[EF\text{-}Tu(1)_{mt}]$ was found from Equation 3 and the previously measured value of K_{GTP} (Table I). $[EF\text{-}Tu(1)_{mt}\cdot Ts_{mt}]$ was then calculated from Equation 14. Equations 11 (with n = 1 and 2), 12, and 15 can be used to find the following expression (Equation 16).

$$[EF\text{-}Tu(2)\cdot Ts_{mt}] = \frac{[EF\text{-}Tu(2)_{mt}]_t}{1 + \left(1 + \frac{[GTP]}{K_{GTP}}\right)\frac{[EF\text{-}Tu(1)_{mt}\cdot GTP]}{[EF\text{-}Tu(1)\cdot Ts_{mt}]}} \qquad \text{(Eq. 16)}$$

which was used to calculate $[EF\text{-}Tu(2)\cdot Ts_{mt}]$. This concentration was then used to calculate $[EF\text{-}Ts_{mt}]$ using Equation 13. The equilibrium dissociation constant K_{Ts} was then found from Equation 11 with n = 1. Equations 12 and 15 also imply the following (Equation 17).

$$[EF\text{-}Tu(2)_{mt}] = \frac{[EF\text{-}Tu(2)_{mt}]_t - [EF\text{-}Tu(2)_{mt}\cdot Ts]}{1 + \frac{[GTP]}{K_{GTP}}} \qquad \text{(Eq. 17)}$$

Using the values of $[EF\text{-}Tu(2)_{mt}]$ obtained from Equation 17, K_{Ts} was found using Equation 11 with n = 2. These values were equivalent to those calculated from Equation 11 with n = 1. The average value of K_{Ts} determined from 11 independent assays was 5.5 ± 1.3 nM.

K_{Ts} for the bovine $EF\text{-}Tu\cdot Ts_{mt}$ complex is close to that of the *E. coli* EF-Tu·Ts complex (2 nM) (2). $EF\text{-}Tu_{mt}$ binds to $EF\text{-}Ts_{mt}$ ~3-fold more tightly than it binds aa-tRNA in the ternary complex in the presence of excess GTP. Compared with the dissociation constants for guanine nucleotides, K_{Ts} is ~2 orders of magnitude lower than K_{GDP} or K_{GTP}. In contrast, in *E. coli,* the EF-Tu·GDP and EF-Tu·Ts complexes have similar dissociation constants.

DISCUSSION

In this study, the equilibrium dissociation constants governing the interaction of bovine $EF\text{-}Tu_{mt}$ with its ligands Phe-tRNA and $EF\text{-}Ts_{mt}$ have been determined. The classical model for the elongation cycle of protein synthesis is based on studies with *E. coli*. Generally, the basic steps in the elongation cycle in mammalian mitochondria are the same as those observed in *E. coli*. However, several of the equilibrium dissociation constants governing the interactions of $EF\text{-}Tu_{mt}$ with its ligands are quite different from those of its *E. coli* counterpart (Table I). In *E. coli*, the dissociation constants for EF-Tu·GDP, EF-Tu·Ts, and EF-Tu·GTP·aa-tRNA are all relatively comparable. In this system, EF-Ts stimulates the dissociation of EF-Tu·GDP, forming the EF-Tu·Ts complex. The EF-Tu·Ts complex is readily dissociated by coupling the formation of the EF-Tu·GTP complex to the formation of the ternary complex. EF-Tu·GDP, EF-Tu·Ts, EF-Tu·GTP, and the ternary complex are all observed as intermediates in the elongation cycle in *E. coli*.

In bovine mitochondria, the elongation cycle also proceeds through these intermediates. However, the binding of guanine nucleotides to $EF\text{-}Tu_{mt}$ is quite weak compared with the binding of $EF\text{-}Ts_{mt}$ or with the binding of aa-tRNA (Table I). Consequently, neither the $EF\text{-}Tu_{mt}$·GDP nor the $EF\text{-}Tu_{mt}$·GTP complex is readily detected in this system. In both *E. coli* and bovine mitochondria, EF-Ts and aa-tRNA bind to EF-Tu with nanomolar affinities. However, for *E. coli* EF-Tu, K_{GDP} is only ~4-fold higher than K_{Ts}, whereas in bovine mitochondria, K_{GDP} is 2 orders of magnitude higher than K_{Ts}. Therefore, in mitochondria, the $EF\text{-}Tu_{mt}$·GDP complex is readily replaced by the formation of $EF\text{-}Tu\cdot Ts_{mt}$.

Although the equilibrium dissociation constants governing the elongation cycle are different in mammalian mitochondria and in prokaryotes, calculations suggest that, under *in vivo* conditions, the ternary complex will be the major form for EF-Tu in both systems (Fig. 7). Using estimated concentrations of the translational components in *E. coli* and the known binding constants (Tables I and II), calculations indicate that 90% of the EF-Tu in *E. coli* will be present in ternary complexes. These estimates are made based on the assumption that aa-tRNAs are free of their cognate aminoacyl-tRNA synthetases and that the system is in equilibrium. There is almost a complete absence of free EF-Tu (<0.01%). Although the volume of a typical liver mitochondrion is similar to that of an *E. coli* cell, the concentrations of the elongation factors in mitochondria are quite different from those found in *E. coli* (Table II). However, despite the dramatic differences in concentrations, the majority of $EF\text{-}Tu_{mt}$ (91%) is still estimated to be present in ternary complexes (Fig. 7). The composition of the different intermediates observed with $EF\text{-}Tu_{mt}$ is thus quite similar to that calculated for the *E. coli* system. These observations suggest that the weak affinity of $EF\text{-}Tu_{mt}$ for GTP and its strong interaction with $EF\text{-}Ts_{mt}$ do not have a negative effect on the availability of the ternary complex for protein biosynthesis. The bacterial and mitochondrial systems both appear to be designed to operate under conditions in which the ternary complex is readily available and is not limiting for translation.

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Interaction of Mitochondrial Elongation Factor Tu with Aminoacyl-tRNA and Elongation Factor Ts

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