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# Interaction of mitochondrial initiation factor 2 with mitochondrial fMet-tRNA

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## ABSTRACT

**The mammalian mitochondrial genome contains a single $tRNA^{Met}$ gene that gives rise to the initiator and elongator $tRNA^{Met}$. It is generally believed that mitochondrial protein synthesis begins with formyl-methionyl-tRNA, which indicates that the formylation of mitochondrial Met-tRNA specifies its participation in initiation through its interaction with initiation factor 2 (IF-2). However, recent studies in yeast mitochondria, suggest that formylation is not required for protein synthesis. In addition, bovine $IF\text{-}2_{mt}$ could replace yeast $IF\text{-}2_{mt}$ in strains that lack fMet-tRNA which suggests that this paradigm may extend to mammalian mitochondria. Here, the importance of the formylation of mitochondrial Met-tRNA for the interaction with $IF\text{-}2_{mt}$ was investigated by measuring the ability of bovine $IF\text{-}2_{mt}$ to bind mitochondrial fMet-tRNA. In direct binding experiments, bovine $IF\text{-}2_{mt}$ has a 25-fold greater affinity for mitochondrial fMet-tRNA than Met-tRNA, using either the native mitochondrial $tRNA^{Met}$ or an *in vitro* transcript of bovine mitochondrial $tRNA^{Met}$. In addition, $IF\text{-}2_{mt}$ will not effectively stimulate mitochondrial Met-tRNA binding to mitochondrial ribosomes, exhibiting a 50-fold preference for fMet-tRNA over Met-tRNA in this assay. Finally, the region of $IF\text{-}2_{mt}$ responsible for the interaction with fMet-tRNA was mapped to the C2 sub-domain of domain VI of this factor.**

## INTRODUCTION

Of the 22 tRNAs encoded in mammalian mitochondrial DNA, there is a single tRNA that represents each amino acid, except for $tRNA^{Ser}$ and $tRNA^{Leu}$, which have 2 isoacceptors each. In mammals, there is no evidence of tRNA import into mitochondria, hence these species represent the complete set of tRNAs available for the translational apparatus. The presence of a single species of $tRNA^{Met}$ is quite unusual. Prokaryotic and eukaryotic cytoplasmic translational systems use two $tRNA^{Met}$ species, one designated for initiation and one for elongation. Even the mitochondria of most lower eukaryotes possess two specialized $tRNA^{Met}$ species. The mammalian mitochondrial translational system, therefore, must have a unique mechanism by which this single $tRNA^{Met}$ species is partitioned between the initiation and elongation phases of protein synthesis. In addition to the single $tRNA^{Met}$ species, mitochondria possess an alternate genetic code in which both AUG and AUA code for methionine (1). A novel modification (5-formyl cytidine) in the first position of the anticodon in $tRNA^{Met}$ may give rise to its ability to decode both AUG and AUA (2,3).

Translational initiation in prokaryotes and mammalian mitochondria generally occurs with fMet-tRNA (4). The gene that encodes the bovine mitochondrial methionyl-tRNA transformylase ($MTF_{mt}$) has been cloned and the protein has been studied biochemically (5–7). The partitioning of $tRNA^{Met}$ into either the initiation phase or the elongation phase of protein synthesis is assumed to occur by a competition between $MTF_{mt}$ and mitochondrial elongation factor Tu ($EF\text{-}Tu_{mt}$) for the Met-$tRNA^{Met}$ (6). Once Met-$tRNA^{Met}$ is formylated by $MTF_{mt}$, it becomes a substrate for mitochondrial initiation factor 2 ($IF\text{-}2_{mt}$) and participates in the initiation phase of protein synthesis. However, if Met-tRNA binds to $EF\text{-}Tu_{mt}$:GTP before it is formylated, it acts as an elongator tRNA. This partitioning mechanism requires that $IF\text{-}2_{mt}$ strongly discriminates against Met-$tRNA^{Met}$ and that $EF\text{-}Tu_{mt}$ preferentially binds Met-$tRNA^{Met}$. For bovine $IF\text{-}2_{mt}$, a strong preference for fMet-tRNA has been demonstrated using both the yeast and *Escherichia coli* initiator $tRNA^{Met}$ species (8). It has also been shown that $EF\text{-}Tu_{mt}$ strongly discriminates against *E.coli* fMet-$tRNA_f^{Met}$ (9). Taken together, these observations suggest that the formylation of mitochondrial $tRNA^{Met}$ governs its identity as either an initiator or an elongator $tRNA^{Met}$.

Recently, however, studies in yeast mitochondria have suggested that formylation of Met-tRNA is not required for mitochondrial protein synthesis. Yeast mutants that lacked the ability to formylate the mitochondrial initiator Met-tRNA had normal protein synthesis, which indicates that formylation is not essential for mitochondrial translation in yeast (10). Further, bovine $IF\text{-}2_{mt}$, at least, when over-expressed in yeast, can support translation in the yeast mitochondria that lacks the ability to formylate Met-$tRNA^{Met}$ (11). These data prompted the suggestion that formylation of Met-tRNA may not be required for translation in mammalian mitochondria. To further understand the role of formylation of Met-tRNA in the yeast mitochondria, yeast $IF\text{-}2_{mt}$ was cloned and characterized biochemically (12). In contrast to previous studies on bovine $IF\text{-}2_{mt}$, yeast $IF\text{-}2_{mt}$ showed a small but significant degree of binding to Met-$tRNA^{Met}$ (12). These observations support the *in vivo* findings that formylation of

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initiator tRNA is not essential in the yeast mitochondrial translational system.

Here, we have investigated the interaction of bovine IF-$2_{mt}$ with mitochondrial fMet-tRNA to determine the role of formylation of mitochondrial Met-tRNA in mammalian mitochondrial protein synthesis.

## MATERIALS AND METHODS

### Materials

The chemicals were purchased from Fisher Scientific or Sigma. [$^{35}$S]Met was obtained from Perkin Elmer Life Science Products. SUPERase•In$^{TM}$ RNase inhibitor was purchased from Ambion. Pyruvate kinase and folinic acid were from Sigma. *E.coli* tRNA was purchased from Roche. *E.coli* $tRNA_f^{Met}$ was partially purified from crude *E.coli* tRNA as described previously (13). The bovine mitochondrial $tRNA^{Met}$ ($mtRNA^{Met}$) transcript and human mitochondrial methionyl-tRNA synthetase ($MetRS_{mt}$) were purified as described previously (14). Crude bovine mitochondrial tRNA was kindly provided by Senyene Eyo Hunter (University of North Carolina at Chapel Hill). IF-$2_{mt}$ and various N- or C-terminal truncated derivatives were expressed and purified as described by A. Spencer and L. Spremulli (submitted for publication). Mitochondrial initiation factor 3 (IF-$3_{mt}$) was kindly provided by Dr Emine Koc (University of North Carolina at Chapel Hill). Bovine liver mitochondria, 55S ribosomes and 28S subunits were prepared as described previously (15,16).

### Expression and purification of bovine $MTF_{mt}$

A plasmid containing the cDNA for bovine $MTF_{mt}$ was kindly provided by Dr Kimitsuna Watanabe (University of Tokyo). The DNA was transformed into *E.coli* BL21(DE3)pLysS cells (Stratagene) for expression. A single colony of cells that harbored the pET15-bovine $MTF_{mt}$ construct was used to inoculate 10 ml of 2YT media (17) that contained 50 μg/ml of ampicillin. This culture was grown at 37°C to saturation and was used to inoculate 2 l of 2YT media (2 × 1 l), which were grown at 37°C to an $A_{600}$ of 0.6–0.8. The expression of bovine $MTF_{mt}$ was induced by the addition of 140 μM isopropyl-β-D-thiogalactopyranoside. Expression was carried out at 18°C for 16 h on a rotary shaker at 150 r.p.m. Following expression, cells were harvested, resuspended in a volume of at least 100 ml of buffer containing 50 mM Tris–HCl (pH 7.6) and pelleted by low speed centrifugation (4000 *g* for 30 min at 4°C). At this point, cells were either fast-frozen and stored at −70°C until future use or treated as described below.

Cell pellets (~9 g) were resuspended in Buffer 1 [50 mM Tris–HCl (pH 7.6), 500 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole, 3 mM 2-mercaptoethanol (BME) and 0.1 mM phenylmethylsulfonyl fluoride] at a ratio of ~10 ml/g cell weight and sonicated at 60 W on ice using 1 s bursts with 4 s cooling periods for 12 min (performed in 3 min intervals). DNase I (5 μg/ml) was added and the lysate was subjected to centrifugation at 38 000 r.p.m. in a Beckman Type 60 rotor for 1 h at 4°C. Following centrifugation, 1.0 ml of Ni-NTA resin (50% slurry in Buffer 1) was added to the supernatant and incubation was carried out at 4°C for 1 h with rocking. The sample was transferred to a polypropylene column and the resin was subsequently washed with ~100 ml Buffer 1. Bovine $MTF_{mt}$ was eluted from the Ni-NTA resin with Buffer 1 containing 220 mM imidazole for 20 min at 4°C. The elution was repeated twice and all eluates were combined before dialyzing the sample against 250 ml of Buffer 2 [20 mM Tris–HCl (pH 7.6), 3 mM BME, 100 mM KCl and 10% glycerol] for 45 min at 4°C with one change of buffer and an additional 45 min of dialysis. This preparation gave a major band on SDS–PAGE with an apparent molecular weight of ~40 kDa and was estimated to be at least 50% pure by Coomassie staining. The sample was used without further purification since it was active and free of nuclease contamination (data not shown).

### Preparation of Met-$tRNA^{Met}$

The large-scale aminoacylation of partially purified *E.coli* $tRNA_f^{Met}$ was carried out essentially as described (18).The large-scale aminoacylation of a bovine mitochondrial $tRNA^{Met}$ transcript was carried out in a reaction (1 ml) containing 50 mM Tris–HCl (pH 7.8), 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.2 mM spermine, 0.2 mg/ml BSA, 8 μM [$^{35}$S]Met (15 000 c.p.m./pmol), 260 μg of partially purified human $MetRS_{mt}$, 100 U SUPERase•In$^{TM}$ and 300 pmol of the bovine $mtRNA^{Met}$ transcript. The reaction was incubated at 37°C for 30 min and the tRNA was purified by two extractions with phenol and one extraction with chloroform. The tRNA was precipitated with ethanol overnight at −20°C and resuspended in 10 mM potassium succinate (pH 6.0). The large-scale aminoacylation of native bovine $mtRNA^{Met}$ was carried out as described above except that the reaction mixtures contained 120 μg of human $MetRS_{mt}$ and 190 μg of crude native bovine mitochondrial tRNA.

### Formylation activity of bovine $MTF_{mt}$

Folinic acid (6.4 mg) was dissolved in 1 ml of 100 mM HCl containing 5 mM DTT and incubated in the dark, at room temperature, for 12 h. The sample was diluted to 10 ml with distilled $H_2O$ to a final concentration of 1.25 mM. The ability of bovine $MTF_{mt}$ to formylate Met-tRNA was monitored using thin layer chromatography (TLC). Formylation reactions (10 μl) contained 20 mM Tris–HCl (pH 7.6), 0.1 mM EDTA, 150 mM KCl, 7 mM $MgCl_2$, 10 mM BME, 10 pmol (1 μM) *E.coli* [$^{35}$S]Met-$tRNA_f^{Met}$ (5000 c.p.m./pmol), 1 μg of partially purified bovine $MTF_{mt}$ and, where indicated, 0.125 mM folinic acid. Following incubation at 37°C for 10 min, 0.5 M NaOH was added to a final concentration of 0.1 M and incubation was continued at 37°C for 30 min. Approximately one-tenth of the reaction mixture was spotted on a Whatman LK2 TLC plate that had previously been run with distilled $H_2O$ and dried. The mobile phase for TLC analysis was butanol/acetic acid/$H_2O$ (4:1:1). The plate was dried and placed on a phosphor screen for at least 12 h. The [$^{35}$S]fMet was visualized by phosphorimaging with a Molecular Dynamics' Storm 840.

To measure the ability of $MTF_{mt}$ to formylate either a bovine mitochondrial Met-$tRNA^{Met}$ transcript or native bovine mitochondrial Met-$tRNA^{Met}$, the formylation reaction was carried out as described above except that it contained 6 pmol (0.6 μM) [$^{35}$S]Met-$tRNA^{Met}$ (5000 c.p.m./pmol)

transcript or 1.5 pmol (0.15 μM) of native mitochondrial [$^{35}$S]Met-tRNA$^{Met}$ (15 000 c.p.m./pmol).

### Preparation of mitochondrial fMet-tRNA$^{Met}$

Large-scale charging and formylation of mitochondrial tRNA$^{Met}$ was performed in a coupled assay essentially as described above for the aminoacylation of mtRNA$^{Met}$ with the following modifications. Reaction mixtures (1 ml) contained 50 mM Tris–HCl (pH 7.8), 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.2 mM spermine, 0.2 mg/ml BSA, 8 μM [$^{35}$S]Met (15 000 c.p.m./pmol), 100 U SUPERase•In$^{TM}$, 0.125 mM folinic acid, 340 μg of partially purified human $MetRS_{mt}$, 50 μg of partially purified bovine $MTF_{mt}$ and 300 pmol of the bovine mtRNA$^{Met}$ transcript. For large-scale aminoacylation and formylation of the native bovine mtRNA$^{Met}$, reaction mixtures (1 ml) were prepared as described above except that they contained 120 μg of partially purified human $MetRS_{mt}$ and 210 μg of crude native mitochondrial tRNA. The reaction mixtures were incubated at 37°C for 30 min and the tRNA was purified from the reaction components as described above. Following purification of the tRNA, an aliquot of the products was analyzed for formylation by using the TLC procedure.

### Interaction of bovine IF-2$_{mt}$ with mitochondrial fMet-tRNA$^{Met}$

The RNase protection assays were carried out essentially as described (19). Reaction mixtures (25 μl) contained 50 mM Tris–HCl (pH 7.8), 2 mM DTT, 7 mM $MgCl_2$, 100 mM $NH_4Cl$, 0.25 mM guanosine 5′-[β,γ-imido]triphosphate (GDPNP), 2 pmol (0.08 μM) bovine mitochondrial [$^{35}$S]fMet-tRNA$^{Met}$ transcript or native bovine mitochondrial [$^{35}$S]fMet-tRNA$^{Met}$ (15 000 c.p.m./pmol) and the indicated amounts of the mature form of IF-2$_{mt}$ (typically 2.8–10 μM). Following incubation at 0°C for 15 min, 5 μg of RNase A was added. Incubation was continued for an additional 30 s at 0°C. The reaction was terminated by the addition of 3 ml of ice-cold 5% trichloroacetic acid (TCA). Incubation at 0°C was continued for 10 min. The precipitate formed was collected on nitrocellulose filters which were washed with 2 × 3 ml cold 5% TCA and dried for 6 min at 100°C. The amount of [$^{35}$S]fMet-tRNA remaining was determined by scintillation counting. A blank representing the amount of [$^{35}$S]fMet-tRNA that remained in the absence of IF-2$_{mt}$ was subtracted from each point (generally <0.1 pmol).

The ability of various derivatives of IF-2$_{mt}$ to bind mitochondrial [$^{35}$S]fMet-tRNA was measured as described above using 3 pmol (0.12 μM) bovine mitochondrial [$^{35}$S]fMet-tRNA transcript and 0.6–1.2 μM of each derivative. For direct comparison of each derivative, the ability of 1.0 μM of each IF-2$_{mt}$ derivative to protect 2.5 pmol (0.1 μM) of the bovine mitochondrial [$^{35}$S]fMet-tRNA transcript was measured. The remainder of the assay was carried out as described above.

### Initiation complex formation on mitochondrial ribosomes with mitochondrial fMet-tRNA$^{Met}$

The ability of IF-2$_{mt}$ to stimulate the binding of mitochondrial [$^{35}$S]fMet-tRNA to 28S subunits was measured in an *in vitro* binding assay essentially as described previously (8,20). Reaction mixtures (50 μl) contained 50 mM Tris–HCl (pH 7.6), 1 mM DTT, 0.1 mM spermine, 35 mM KCl, 7.5 mM $MgCl_2$, 0.25 mM GDPNP, 5 μg poly(A,U,G), 10 U SUPERase•In$^{TM}$, 2.5 pmol (0.05 μM) 28S subunits, 0.13–3 pmol (2.6–60 nM) of mitochondrial [$^{35}$S]fMet-tRNA (15 000 c.p.m./pmol) and the indicated amounts of IF-2$_{mt}$ (3–7 pmol, 0.06–0.14 μM). Following incubation of the reactions at 27°C for 20 min, 3 ml of ice-cold buffer [50 mM Tris–HCl (pH 7.6), 35 mM KCl and 7.5 mM $MgCl_2$] was added and the reactions were filtered through a nitrocellulose membrane (Millipore type HA). Each filter was washed twice with 3 ml of the above buffer and dried at 100°C for 6 min. The amount of [$^{35}$S]fMet-tRNA bound to the ribosome was determined by liquid scintillation counting. A blank representing the amount of [$^{35}$S]fMet-tRNA that bound to the 28S subunit in the absence of IF-2$_{mt}$ at each concentration of fMet-tRNA was subtracted from each value (~0.01–0.03 pmol).

The ability of IF-2$_{mt}$ to promote the binding of mitochondrial fMet-tRNA to mitochondrial 55S ribosomes was carried out essentially as described above, except that reaction mixtures (50 μl) contained 50 mM Tris–HCl (pH 7.6), 1 mM DTT, 0.1 mM spermine, 35 mM KCl, 7.5 mM $MgCl_2$, 0.25 mM GTP, 1.25 mM phosphoenolpyruvate (PEP), 0.5 U pyruvate kinase, 5 μg poly(A,U,G), 10 U SUPERase•In$^{TM}$, 20 pmol (0.4 μM) mitochondrial IF-3, 3.0–3.5 pmol (0.06–0.07 μM) 55S ribosomes, 0.125–1.5 pmol (2.5–30 nM) of either native or the transcript of bovine mitochondrial [$^{35}$S]fMet-tRNA (15 000 c.p.m./pmol) and the indicated amounts of IF-2$_{mt}$ (4–14 pmol, 0.08–0.28 μM). The reactions were incubated at 27°C for 20 min and treated as described above. A blank representing the amount of [$^{35}$S]fMet-tRNA binding to the 55S ribosome in the absence of IF-2$_{mt}$ (~0.01–0.04 pmol) at each concentration of fMet-tRNA has been subtracted from each value.

## RESULTS

### Ability of bovine $MTF_{mt}$ to formylate mitochondrial tRNA$^{Met}$

The ability of bovine $MTF_{mt}$ to formylate various Met-tRNA$^{Met}$ substrates was tested to assess whether the enzyme was active with the transcript of the tRNA$^{Met}$ gene as well as with the native tRNA. Following the reaction, the ester bond on the tRNA was hydrolyzed by NaOH, releasing the radiolabeled amino acid that was analyzed for formylation by TLC, which clearly separates Met and fMet. The primary determinant for substrate recognition by bovine $MTF_{mt}$ is the amino acid methionine, and previous studies have shown that *E.coli* Met-tRNA$_f^{Met}$ is efficiently formylated by the mitochondrial enzyme (6,7). *E.coli* Met-tRNA was initially used to test the experimental approach for monitoring the formylation reaction, before using the mitochondrial Met-tRNA. As expected, bovine $MTF_{mt}$ efficiently formylated the heterologous *E.coli* Met-tRNA$_f^{Met}$ substrate in the presence of the formyl group donor (Figure 1, lanes 1 and 2) (7). Yeast [$^{35}$S]fMet-tRNA was used as a control for the location of fMet in these experiments and contains a small amount of methionine oxidation products that have a lower $R_f$ value than methionine (Figure 1, lane 3). In the presence of the formyl group donor, bovine $MTF_{mt}$ effectively formylated the *in vitro* transcript of mitochondrial Met-tRNA$^{Met}$ (Figure 1, lanes 4 and 5) and the native mitochondrial Met-tRNA$^{Met}$

(Figure 1, lanes 6 and 7). Under the conditions used, close to 100% of the mitochondrial Met-tRNA could be formylated.

### Interaction of IF-$2_{mt}$ with mitochondrial fMet-$tRNA^{Met}$

The ability of bovine IF-$2_{mt}$ to bind formylated and unformylated Met-tRNA in solution was assessed by measuring the amount of fMet-tRNA protected from hydrolysis by RNase A in the presence of increasing amounts of IF-$2_{mt}$. The binding of fMet-tRNA and Met-tRNA to bovine IF-$2_{mt}$ was determined both for the *in vitro* transcript and for the native bovine mitochondrial fMet-$tRNA^{Met}$. Bovine IF-$2_{mt}$ clearly has a much higher affinity for fMet-$tRNA^{Met}$ and discriminates against Met-tRNA when either the transcript or the native mitochondrial $tRNA^{Met}$ is used (Figure 2). The amount of protection achieved with the transcript is slightly higher than that obtained with the native fMet-$tRNA^{Met}$, which indicates that the modified nucleotides present in the native bovine $mtRNA^{Met}$ do not play a role in the recognition of mitochondrial fMet-$tRNA^{Met}$ by bovine IF-$2_{mt}$. These modifications include two pseudouridines and the $f^5C$ that is located in the first position of the anticodon. The lower degree of binding to the native mitochondrial fMet-$tRNA^{Met}$ could also indicate that the presence of a large excess of non-specific mitochondrial tRNA interferes with the binding to IF-$2_{mt}$. Nonetheless, the binding of mitochondrial fMet-tRNA to bovine IF-$2_{mt}$ was at least 50-fold greater than the binding observed with mitochondrial Met-tRNA.

The strong preference of IF-$2_{mt}$ for fMet-tRNA contrasts with the weaker, 4-fold, preference for fMet-tRNA observed with yeast IF-$2_{mt}$ (12). This measurement, however, was done in a filter binding assay in the absence of magnesium and may not reflect a biologically relevant interaction (12). Similar to bovine IF-$2_{mt}$, yeast IF-$2_{mt}$ did not protect the unformylated Met-tRNA in a RNase protection assay (12). It should also be noted that GDPNP did not affect the binding of fMet-tRNA to bovine IF-$2_{mt}$. The same observation was made for the interaction of *E.coli* IF-2 with fMet-tRNA (21).

Bovine IF-$2_{mt}$ is organized into four domains and contains the equivalent of domains III–VI of *E.coli* IF-2 (Figure 3A) (22). To determine the domain(s) of bovine IF-$2_{mt}$ involved in the interaction with mitochondrial fMet-tRNA, the ability of a number of IF-$2_{mt}$ derivatives truncated at the N- and/or C-terminus to bind mitochondrial fMet-tRNA was measured in the RNase protection assay. Initially, the ability of the

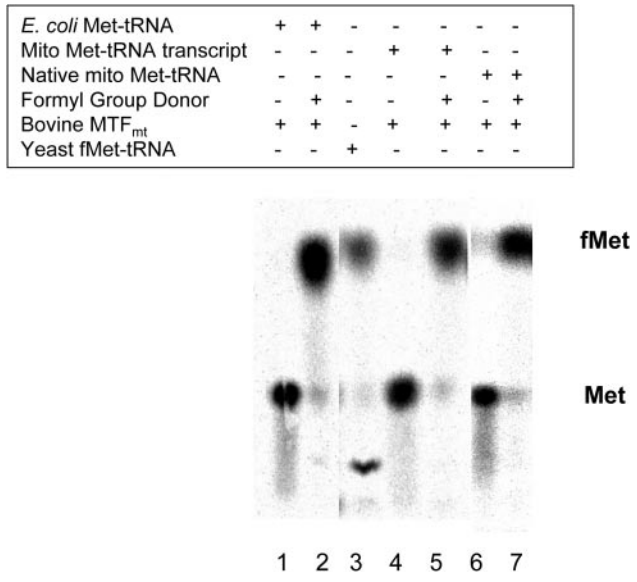


**Figure 1.** TLC analysis of the formylation of Met-tRNA by bovine $MTF_{mt}$. Formylation reactions contained bovine $MTF_{mt}$ and either *E.coli* [$^{35}$S]Met-tRNA or mitochondrial [$^{35}$S]Met-tRNA as indicated. The numbers in parentheses indicate the amount applied in each lane in the TLC analysis. Lane 1: *E.coli* [$^{35}$S]Met-tRNA (1 pmol) in the absence of a formyl group donor. Lane 2: *E.coli* [$^{35}$S]Met-tRNA (1 pmol) in the presence of a formyl group donor. Lane 3: yeast [$^{35}$S]fMet-tRNA (1 pmol) prepared as described in (18) as a marker for fMet. Lane 4: mitochondrial [$^{35}$S]Met-tRNA transcript (1 pmol) in the absence of a formyl group donor. Lane 5: mitochondrial [$^{35}$S]Met-tRNA transcript (1 pmol) in the presence of a formyl group donor. Lane 6: native mitochondrial [$^{35}$S]Met-tRNA (0.2 pmol) in the absence of a formyl group donor. Lane 7: native mitochondrial [$^{35}$S]Met-tRNA (0.2 pmol) in the presence of a formyl group donor.

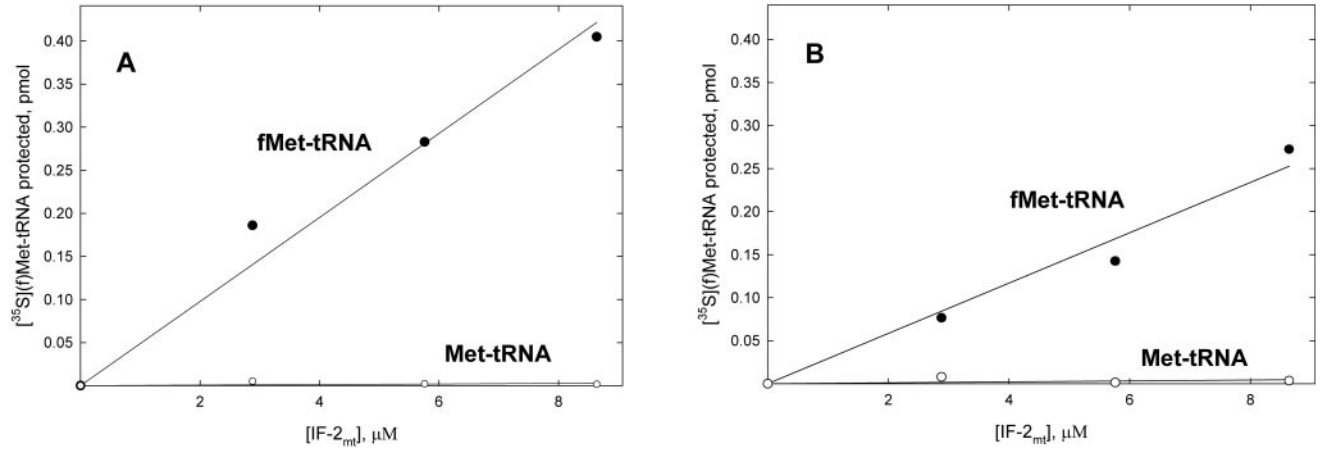


**Figure 2.** Interaction of bovine IF-$2_{mt}$ with mitochondrial fMet-tRNA. The ability of bovine IF-$2_{mt}$ to protect mitochondrial fMet-tRNA from hydrolysis by RNase A was measured as described in Materials and Methods. A blank representing the amount of [$^{35}$S]fMet-tRNA that remained in the absence of IF-$2_{mt}$ was subtracted from each point. Data were fit by linear least-squares analysis. (**A**) Protection assays contained 2 pmol of either mitochondrial [$^{35}$S]fMet-tRNA transcript (closed circles) or 2 pmol of mitochondrial [$^{35}$S]Met-tRNA transcript (open circles) and the indicated amounts of IF-$2_{mt}$. (**B**) Protection assays contained 2 pmol of either native mitochondrial [$^{35}$S]fMet-tRNA (closed circles) or 2 pmol native mitochondrial [$^{35}$S]Met-tRNA (open circles) and the indicated amounts of IF-$2_{mt}$.

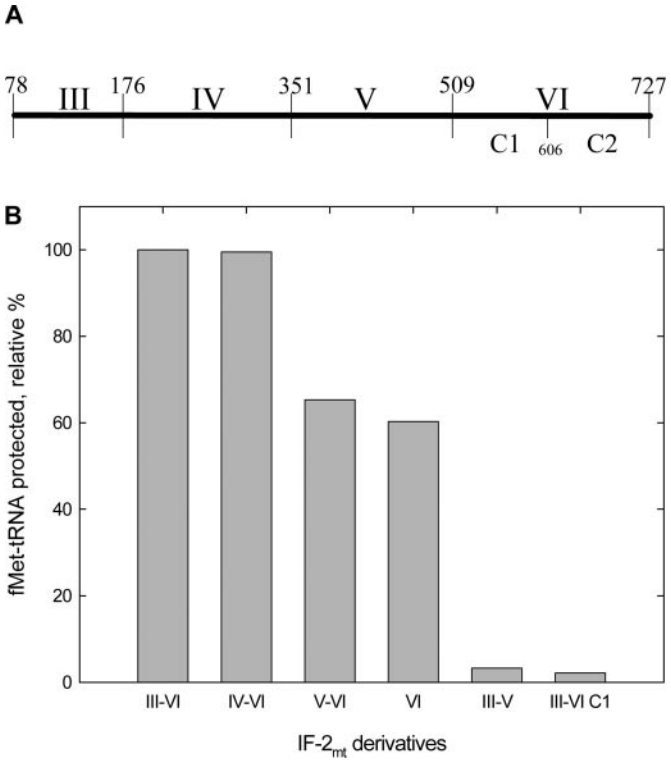


**Figure 3.** Binding of various IF-$2_{mt}$ derivatives to mitochondrial fMet-tRNA. (**A**) Domain organization of bovine IF-$2_{mt}$ and the derivatives used to determine the domain(s) of IF-$2_{mt}$ responsible for the interaction with fMet-tRNA. The domains have been assigned based on the *E.coli* IF-2 nomenclature (22) and the amino acid residues that encompasses each domain are indicated. (**B**) The relative percentage of fMet-tRNA protected was calculated based on the amount of [$^{35}$S]fMet-tRNA (2.5 pmol input, 0.1 μM) protected by 1.0 μM of each derivative. The percentage of fMet-tRNA protected by the full-length IF-$2_{mt}$ was set at 100% (∼5% of input tRNA) to facilitate comparison. The amount of [$^{35}$S]fMet-tRNA that remained in the absence of IF-$2_{mt}$ has been subtracted from each value (0.05 pmol).

various derivatives to protect fMet-tRNA was determined for several concentrations of IF-$2_{mt}$. The protection of [$^{35}$S]fMet-tRNA was linear for each derivative over the concentration range tested (0.6–1.2 μM) (data not shown). To simplify the analysis, the ability of each derivative to bind mitochondrial fMet-tRNA was measured at a single concentration of IF-$2_{mt}$ (1.0 μM) (Figure 3B). There was no significant difference in the ability of either the domains III–VI or the domains IV–VI derivative to bind fMet-tRNA (Figure 3B). Thus, domain III does not appear to play a role in the binding of fMet-tRNA to IF-$2_{mt}$. Removal of domain IV, however, caused a 40% reduction in the ability of IF-$2_{mt}$ to bind fMet-tRNA. The isolated domain VI alone was capable of binding fMet-tRNA (Figure 3B), although it was somewhat less active than the full-length factor. The decrease in binding observed in both the domains V and VI, and domain VI derivatives does not imply a direct role of domain IV in the interaction with fMet-tRNA, but presumably reflects the influence of domain IV on the overall conformation of IF-$2_{mt}$ (A.C. Spencer and L.L. Spremulli, unpublished data). Both derivatives lacking the C2 sub-domain of domain VI (domains III–V and domains III–VI C1) were completely inactive in binding fMet-tRNA. Thus, it appears that domain VI of IF-$2_{mt}$ is responsible for the interaction of IF-$2_{mt}$ with fMet-tRNA. These data indicate that, like prokaryotic IF-2, domain VI C2 is directly responsible for the interaction with fMet-tRNA (23–26).

### Initiation complex formation on mitochondrial ribosomes with mitochondrial fMet-tRNA

The ability of bovine IF-$2_{mt}$ to stimulate the binding of mitochondrial fMet-tRNA to mitochondrial 28S subunits was measured in the presence of poly(A,U,G) and a non-hydrolyzable analogue of GTP, GDPNP (Figure 4). In the context of the small ribosomal subunit and the synthetic mRNA, bovine IF-$2_{mt}$ prefers the formylated mitochondrial Met-tRNA transcript up to 10-fold over the unformylated Met-tRNA (Figure 4A). Similarly, there is also a clear discrimination of the native mitochondrial fMet-tRNA over the native Met-tRNA by bovine IF-$2_{mt}$ in the context of 28S subunits (Figure 4B). Bovine IF-$2_{mt}$ prefers the native mitochondrial fMet-tRNA over Met-tRNA by ∼25-fold. When assayed on *E.coli* 30S subunits, yeast IF-$2_{mt}$ showed only a 6- to7-fold preference for fMet-tRNA over Met-tRNA (12).

The ability of bovine IF-$2_{mt}$ to stimulate fMet-tRNA binding to mitochondrial 55S ribosomes was measured in the presence of poly(A,U,G), GTP and mitochondrial IF-3 (Figure 5). Again, bovine IF-$2_{mt}$ clearly prefers both the transcript and the native mitochondrial fMet-tRNA$^{Met}$ by ∼20-fold and 50-fold, respectively (Figure 5A and B).

## DISCUSSION

In contrast to recent studies in yeast mitochondria, it is clear that bovine IF-$2_{mt}$ strongly prefers mitochondrial fMet-tRNA over Met-tRNA. These observations support the idea that protein synthesis in mammalian mitochondria does in fact involve fMet-tRNA. Earlier support of this idea comes from the analysis of the N-terminal amino acid of mitochondrially synthesized proteins, which were found to contain formylmethionine, as well as the requirement of $N^{10}$-formyl tetrahydrofolate for mitochondrial protein synthesis (4,10). In addition, the discovery of Met-tRNA transformylase activity in bovine mitochondria gave further support to the belief that fMet-tRNA initiates mitochondrial protein synthesis, as it does in prokaryotic systems (6).

The observations made here are in contrast to those made *in vivo* in yeast where it has been observed that bovine IF-$2_{mt}$ can support protein synthesis in the absence of formylated Met-tRNA (11). One explanation for this apparent discrepancy is that bovine IF-$2_{mt}$ was over-expressed in these cells that perhaps compensated for its low affinity for Met-tRNA. A similar increase in the levels of IF-2 was observed in an *E.coli* mutant capable of initiating protein synthesis without formylated initiator tRNA (27,28). However, at physiological levels of IF-$2_{mt}$, the pathway of translational initiation in mitochondria would occur via fMet-tRNA.

Biochemical data on bacterial IF-2 emphasizes the importance of the formyl group for the recognition of the initiator tRNA by IF-2. Bacterial IF-2 selects the initiator tRNA to a significant extent on the basis of the fMet moiety (29,30). Studies on *Thermus thermophilus* IF-2 also showed that fMet-AMP could be used as a minimal substrate for IF-2 (24). A portion of IF-2, the C2 sub-domain (Figure 3A), binds

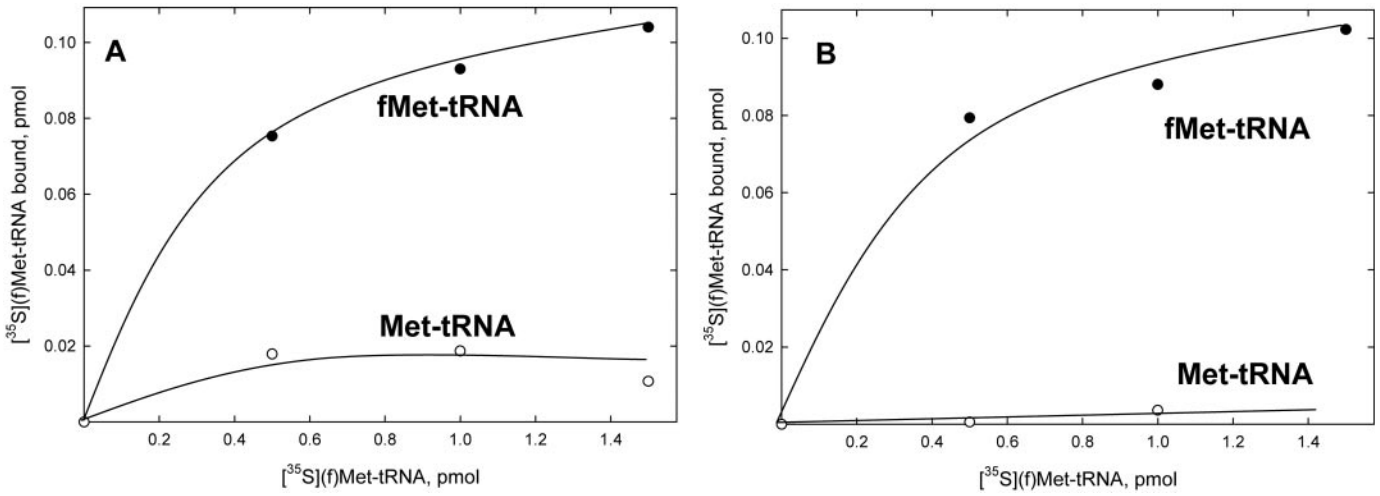


**Figure 4.** Binding of mitochondrial fMet-tRNA to mitochondrial 28S subunits in the presence of IF-$2_{mt}$. (**A**) Reaction mixtures contained 2.5 pmol (0.05 μM) 28S subunits, 3 pmol (0.06 μM) bovine IF-$2_{mt}$ and the indicated amounts of mitochondrial [$^{35}$S]fMet-tRNA transcript (closed circles) or mitochondrial [$^{35}$S]Met-tRNA transcript (open circles). Curves represent a visual best-fit of the data. (**B**) Reaction mixtures contained 2.5 pmol (0.05 μM) 28S subunits, 7 pmol (0.14 μM) IF-$2_{mt}$ and the indicated amounts of native mitochondrial [$^{35}$S]fMet-tRNA (closed circles) or native mitochondrial [$^{35}$S]Met-tRNA (open circles).

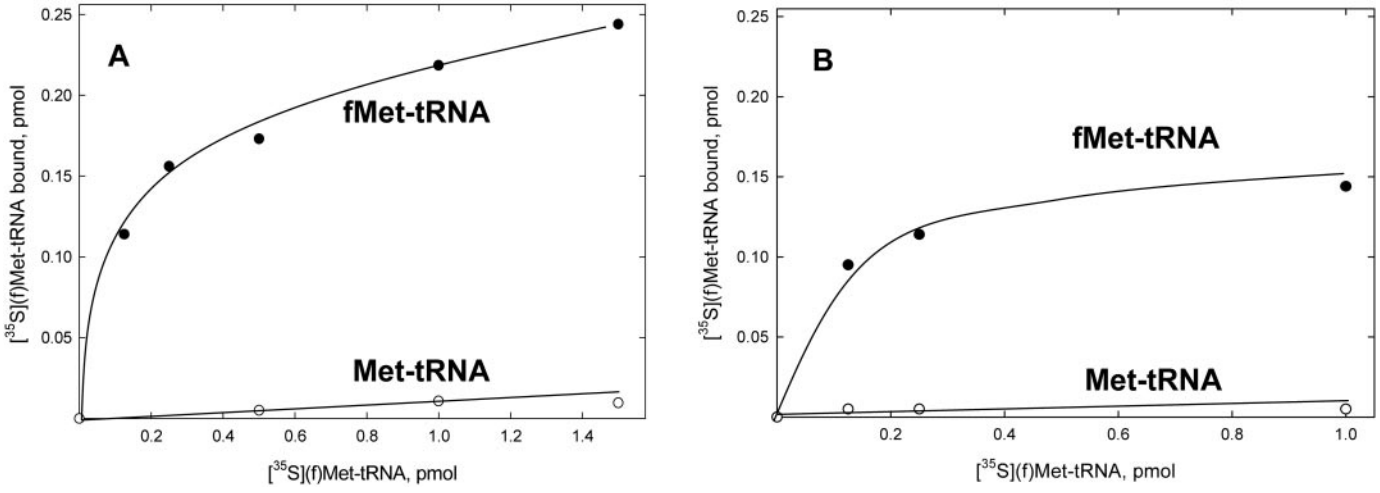


**Figure 5.** Binding of mitochondrial fMet-tRNA to mitochondrial 55S ribosomes in the presence of bovine IF-$2_{mt}$. (**A**) Reaction mixtures contained 3.5 pmol (0.07 μM) 55S ribosomes, 14 pmol (0.3 μM) IF-$2_{mt}$ and the indicated amounts of mitochondrial [$^{35}$S]fMet-tRNA transcript (closed circles) or mitochondrial [$^{35}$S]Met-tRNA transcript (open circles). Curves represent a visual best-fit of the data. (**B**) Reaction mixtures contained 3 pmol (0.06 μM) 55S ribosomes, 4 pmol (0.08 μM) IF-$2_{mt}$ and the indicated amounts of native mitochondrial [$^{35}$S]fMet-tRNA (closed circles) or native mitochondrial [$^{35}$S]Met-tRNA (open circles).

fMet-tRNA with the same affinity as full-length IF-2 (25). The structure of the C2 sub-domain of domain VI of *Bacillus stearothermophilus* IF-2 has been solved using NMR (31) and folds into a β-barrel similar to domain II of EF-Tu. The combination of structural information and mutagenesis data on residues near the surface of IF-2 contributed to the mapping of the binding site for fMet-tRNA on IF-2 (32). Several conserved amino acid residues in *B.stearothermophilus* IF-2 are important for fMet-tRNA binding (32). These include Arg-700 which lies close to the formyl group, Lys-699 and Lys-702 which are necessary for fMet-tRNA binding and Cys-668 and Gly-715 which are thought to create a groove to accommodate the Met side chain (32). The majority of these residues are conserved in IF-$2_{mt}$ indicating a commonality in the preferential binding to fMet-tRNA for both bacterial and mitochondrial IF-2.

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