



# Roles of the N- and C-Terminal Domains of Mammalian Mitochondrial Initiation Factor 3 in Protein Biosynthesis

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Bacterial initiation factor 3 (IF3) is organized into N- and C-domains separated by a linker. Mitochondrial IF3 (IF3<sub>mt</sub>) has a similar domain organization, although both domains have extensions not found in the bacterial factors. Constructs of the N- and C-domains of IF3<sub>mt</sub> with and without the connecting linker were prepared. The  $K_d$  values for the binding of full-length IF3<sub>mt</sub> and its C-domain with and without the linker to mitochondrial 28S subunits are 30, 60, and 95 nM, respectively, indicating that much of the ribosome binding interactions are mediated by the C-domain. However, the N-domain binds to 28S subunits with only a 10-fold lower affinity than full-length IF3<sub>mt</sub>. This observation indicates that the N-domain of IF3<sub>mt</sub> has significant contacts with the protein-rich small subunit of mammalian mitochondrial ribosomes. The linker also plays a role in modulating the interactions between the 28S subunit and the factor; it is not just a physical connector between the two domains. The presence of the two domains and the linker may optimize the overall affinity of IF3<sub>mt</sub> for the ribosome. These results are in sharp contrast to observations with *Escherichia coli* IF3. Removal of the N-domain drastically reduces the activity of IF3<sub>mt</sub> in the dissociation of mitochondrial 55S ribosomes, although the C-domain itself retains some activity. This residual activity depends significantly on the linker region. The N-domain alone has no effect on the dissociation of ribosomes. Full-length IF3<sub>mt</sub> reduces the binding of fMet-tRNA to the 28S subunit in the absence of mRNA. Both the C-terminal extension and the linker are required for this effect. IF3<sub>mt</sub> promotes the formation of a binary complex between IF2<sub>mt</sub> and fMet-tRNA that may play an important role in mitochondrial protein synthesis. Both domains play a role promoting the formation of this complex.

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## Introduction

Mitochondria have their own genomes, which, in mammals, contain only 16,000 base pairs of DNA. This DNA encodes 13 proteins that are synthesized within the organelle. All of these proteins are inserted into the inner membrane and are involved in oxidative phosphorylation. The synthesis and

assembly of these polypeptides in the inner membrane are not well understood.

The mammalian mitochondrial translation system has a number of interesting features. These mitochondrial ribosomes (55S) have a higher protein content than bacterial ribosomes, while their rRNAs are considerably truncated. The 55S particle consists of a 28S small subunit and a 39S large subunit.<sup>1</sup> Cryo-electron microscopy indicates that the core of the mitochondrial ribosome is decorated with many proteins that have no bacterial homologs.<sup>2</sup> Therefore, the interactions of these ribosomes with mRNA, tRNA, and translational factors are expected to show interesting differences compared to those occurring in bacterial systems.

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Abbreviations used: IF3, initiation factor 3; IF3<sub>mt</sub>, mitochondrial IF3; EDTA, ethylenediaminetetraacetic acid; TEV, tobacco etch virus; RU, resonance units.

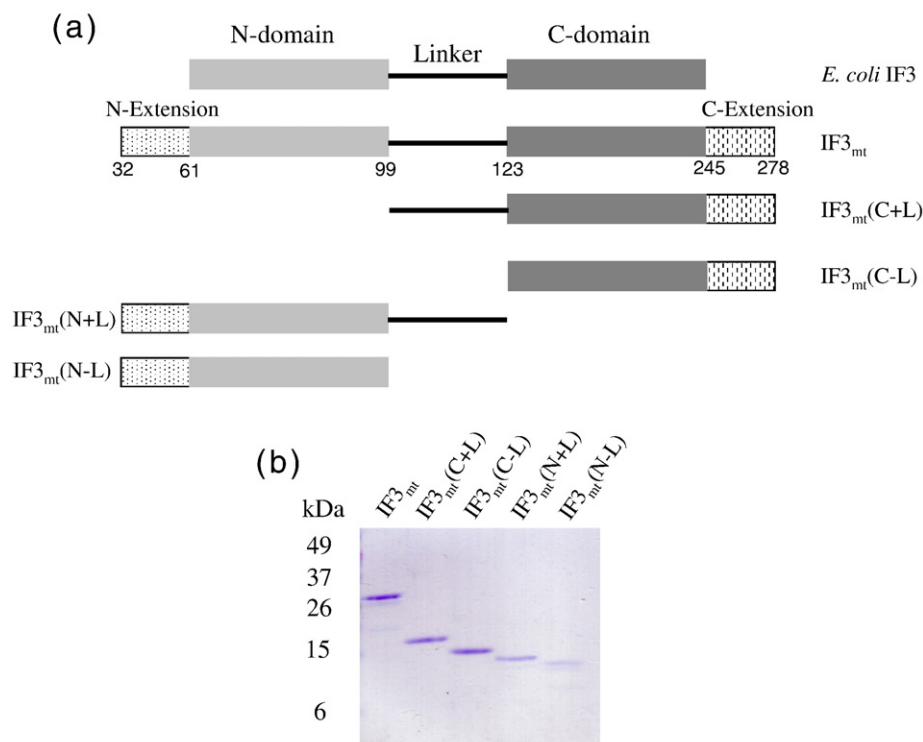
Two initiation factors have been found in mammalian mitochondria. Mitochondrial initiation factor 2 (IF2<sub>mt</sub>) promotes the binding of fMet-tRNA to the ribosome. Recently, Gaur *et al.* provided evidence that mammalian IF2<sub>mt</sub> serves the functions not only of IF2 but also of IF1 in mitochondria.<sup>3</sup> Human mitochondrial initiation factor 3 (IF3<sub>mt</sub>) has been cloned and expressed in *Escherichia coli*. It has both similarities and differences with its bacterial counterparts.

The activities of *E. coli* IF3 are well established.<sup>4,5</sup> This factor plays several roles in initiation complex formation including functioning as an anti-association (or dissociating) agent for the ribosomal subunits, enhancing codon-anticodon interactions at the P-site, and increasing the rate of dissociation of noncanonical 30S initiation complexes.<sup>6-10</sup> Recent kinetic studies have shown that the joining of the 50S subunit to 30S initiation complexes and the dissociation of IF3 from the small subunit are strongly regulated by the codon used for initiation.<sup>11</sup> This result is supported by earlier investigations.<sup>12</sup>

*E. coli* IF3 consists of two domains (N-domain and C-domain) joined by a flexible linker (Fig. 1). The isolated C-domain of *E. coli* IF3 binds to 30S subunits

but with a 100-fold lower affinity than full-length IF3.<sup>9</sup> The isolated N-domain does not bind detectably to 30S subunits and has no known function,<sup>9</sup> although it clearly enhances the interaction of the C-domain with the small subunit. Recent studies indicate that the N-domain may modulate the association and dissociation of IF3 from the 30S subunit through a fluctuating interaction with the neck region of the subunit.<sup>13</sup> It has also been reported that the N-terminal domain may provide a proofreading function since mutation of Tyr75 or iodination of Tyr70 in the N-domain results in a factor that could not dissociate incorrect initiation complexes.<sup>14,15</sup>

IF3<sub>mt</sub> has a central region with homology to the bacterial factors with additional N-terminal and C-terminal extensions (Fig. 1a). The roles of IF3<sub>mt</sub> and its terminal extensions in initiation complex formation have been investigated.<sup>16-18</sup> These studies suggest that the C-terminal extension reduces the affinity of the factor for 39S subunits, thus promoting proper subunit joining. In the present study, we have examined the properties of the isolated N- and C-domains of IF3<sub>mt</sub> carrying the extensions on mitochondrial protein synthesis.



**Fig. 1.** Domain organization of bacterial IF3 and human IF3<sub>mt</sub> and its truncated C- and N-domains with and without the linker. (a) Pictorial representation of the domain structure of *E. coli* IF3 and human IF3<sub>mt</sub> and the four truncated derivatives used in this study. IF3<sub>mt</sub> and its C-terminal domain with the linker IF3<sub>mt</sub>(C+L) and without the linker IF3<sub>mt</sub>(C-L) and the N-terminal domain with the linker IF3<sub>mt</sub>(N+L) and without the linker IF3<sub>mt</sub>(N-L) region are shown. The numbers indicate the amino acid residues in IF3<sub>mt</sub>, with the first residue of the signal sequence designated as residue 1. The import signal was removed in the preparation of the full-length factor and the various constructs. (b) SDS-PAGE analysis of the IF3<sub>mt</sub> derivatives prepared following expression in *E. coli*. The amounts of the proteins used were IF3<sub>mt</sub>, 0.7 µg; IF3<sub>mt</sub>(C+L), 0.45 µg; IF3<sub>mt</sub>(C-L), 0.42 µg; IF3<sub>mt</sub>(N+L), 0.39 µg; and IF3<sub>mt</sub>(N-L), 0.23 µg. The gel was stained with Coomassie Blue.

## Results

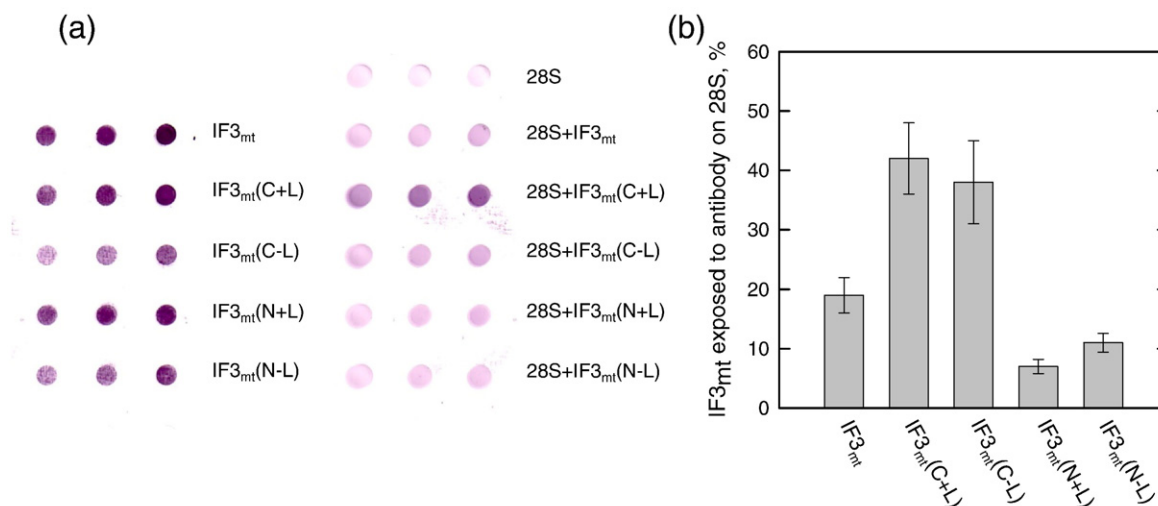
### Interactions of isolated N- and C-domains of IF3<sub>mt</sub> with small ribosomal subunits and the role of the linker region in this interaction

IF3<sub>mt</sub> has ~20–25% identity to bacterial IF3. Unlike bacterial IF3, mammalian IF3<sub>mt</sub> contains N-terminal and C-terminal extensions surrounding the homologous N- and C-domains (Fig. 1a). To explore the role of the extended N- and C-domains and the linker of IF3<sub>mt</sub> in binding to 28S subunits and initiation complex formation, we prepared four derivatives (Fig. 1a). Two His<sub>6</sub>-tagged derivatives were generated for the N-domain, one with the linker, IF3<sub>mt</sub>(N+L), and the other without the linker, IF3<sub>mt</sub>(N–L). Similarly, two derivatives of the C-domain were generated, one with the linker, IF3<sub>mt</sub>(C+L), and the other without the linker, IF3<sub>mt</sub>(C–L) (Fig. 1b).

The interaction of purified IF3<sub>mt</sub>(N+L), IF3<sub>mt</sub>(N–L), IF3<sub>mt</sub>(C+L), and IF3<sub>mt</sub>(C–L) with 28S subunits was determined by Microcon-100 centrifugation followed by Western blotting using antibodies against IF3<sub>mt</sub><sup>18</sup>. For quantification, calibration curves were generated for each domain because the antigenic responses of the various domains were quite different (Fig. 2 and Supplementary Fig. S1). Full-length IF3<sub>mt</sub> gives a strong antigenic response with less than 0.2 pmol of

factor being readily detectable. Good responses are also observed with IF3<sub>mt</sub>(C+L) and IF3<sub>mt</sub>(N+L) using about 2.5-fold more factor. However, removal of the linker in the IF3<sub>mt</sub>(N–L) and IF3<sub>mt</sub>(C–L) derivatives reduces the antigenicity of the factor and 6-fold more protein is required for easy detection (Fig. 2a). These observations suggest that the linker region contains major antigenic determinants.

The binding of IF3<sub>mt</sub> or its derivatives to the 28S subunit leads to a strong quenching (about 80%) of the antigenic response, indicating that a number of the epitopes on the factor are hidden by interactions with the ribosome (Fig. 2a and b). This quenching can be overcome by treatment of the samples with ethylenediaminetetraacetic acid (EDTA), which damages the structure of the small subunit releasing the factor. The antigenicity of the C-domain with or without the linker is also reduced by binding to the small subunit although to a lower extent than observed with the full-length factor. The epitopes in the N-domain with or without the linker are almost entirely obscured by binding to the 28S subunit, indicating that this region of IF3<sub>mt</sub> makes significant contacts with the small subunit. Both the linker and the N-domain appear to be sequestered from the antibodies when this region of IF3<sub>mt</sub> is bound to the small subunit. This observation is surprising since the linker is quite exposed and can be cleaved by trypsin when *E. coli* IF3 is bound to the



**Fig. 2.** Detection of IF3<sub>mt</sub> and its derivatives using antibodies in the presence and absence of 28S subunits. The accessibility of IF3<sub>mt</sub> and its N- and C-domains to antibody in the presence of 28S subunits was studied using the dot blot method. (a) Representative dot blots are shown: the left panel corresponds to protein alone and the right panel corresponds to 28S+IF3<sub>mt</sub>. Reaction mixtures (100  $\mu$ L) contained where indicated 5 pmol of 28S subunits and IF3<sub>mt</sub> (0.16, 0.25, and 0.41 pmol), IF3<sub>mt</sub>(C+L) (0.8, 1.64, and 2.5 pmol), IF3<sub>mt</sub>(C–L) (2.2, 4.4, and 6.6 pmol), IF3<sub>mt</sub>(N+L) (0.82, 1.7, and 2.5 pmol), and IF3<sub>mt</sub>(N–L) (2, 4, and 6 pmol). (b) Plot of the reduction in the antigenic response of the various IF3<sub>mt</sub> derivatives in the presence of 28S subunits. Standard curves were generated at different concentrations of protein in the absence of 28S subunits and fitted with an exponential raise equation [ $y = a(1 - e^{-bx})$ ] where  $y$  is the number of pixels,  $x$  is the concentration of IF3<sub>mt</sub>, and 100% exposure to antibody is assumed in the linear part of the curve. In the presence of 28S subunits, plots of pixel intensity (28S+IF3<sub>mt</sub>) as a function of protein concentration are linear at lower concentrations of protein. Pixel values were determined at low concentrations of protein to maximize the amount of the protein bound to 28S subunits (IF3<sub>mt</sub>:28S ratios of 1:10 or less). The relative exposure of the epitopes of IF3<sub>mt</sub> and its derivatives to the antibody was calculated from the ratio of the pixels of IF3<sub>mt</sub> obtained in the presence of 28S subunits divided by the pixels observed in the presence of protein alone.

30S subunit.<sup>19</sup> The epitopes on all of these derivatives are exposed upon EDTA treatment of the samples, allowing the antigenic detection of the factors in the experiments to quantitate their binding to the small subunit. Individual calibration curves were generated for each derivative in the presence of EDTA, permitting accurate quantitation of the amount of factor bound to the small subunit.

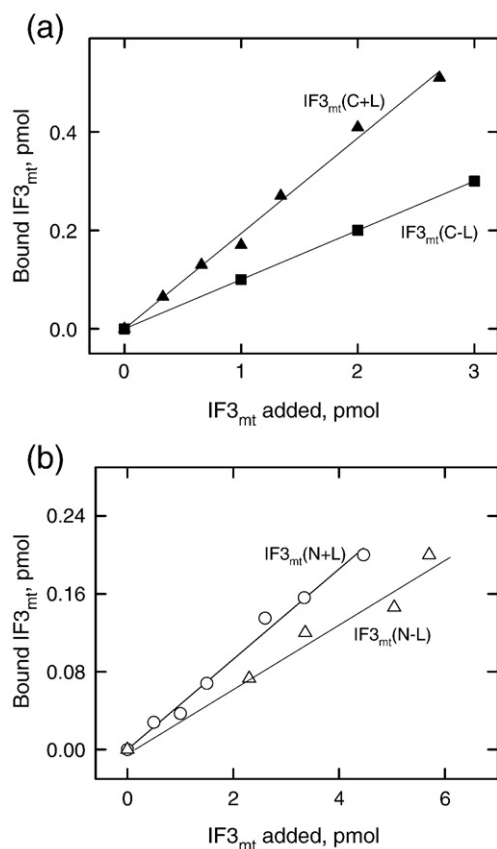
For determination of the apparent equilibrium dissociation constants, different amounts of the IF3<sub>mt</sub> domain derivatives were incubated with a fixed amount of 28S subunits, and the amounts of the derivatives bound to 28S subunits were calculated (Fig. 3), permitting the documentation of the  $K_d$  values governing the interactions (Table 1). The  $K_d$  values are 30, 60, and 95 nM for full-length IF3<sub>mt</sub>, IF3<sub>mt</sub>(C+L), and IF3<sub>mt</sub>(C-L), respectively. Unlike the C-domain of *E. coli* IF3, the isolated C-domain of IF3<sub>mt</sub>

**Table 1.** The apparent equilibrium dissociation constants ( $K_d$ ) for the interaction of IF3<sub>mt</sub> and its C- and N-domains to 28S subunits

IF3 <sub>mt</sub> derivative used	$K_d$ (nM)
IF3 <sub>mt</sub>	30 ± 4 <sup>a</sup>
IF3 <sub>mt</sub> (C+L)	60 ± 5
IF3 <sub>mt</sub> (C-L)	95 ± 13
IF3 <sub>mt</sub> (N+L)	240 ± 40
IF3 <sub>mt</sub> (N-L)	390 ± 60

The apparent  $K_d$  was estimated from the following equation:  $K_d = [IF3_{mt}]_{free}[28S]_{free}/[IF3_{mt} \cdot 28S]$ , where  $[IF3_{mt}]_{free}$  indicates the concentration of free IF3<sub>mt</sub>,  $[28S]_{free}$  indicates the free 28S subunits, and  $[IF3_{mt} \cdot 28S]$  indicates the concentration of IF3<sub>mt</sub> bound to 28S subunits.

<sup>a</sup> Value is taken from a previous publication.<sup>18</sup>



**Fig. 3.** Interaction of the C- and N-domains of IF3<sub>mt</sub> with small ribosomal subunits (28S). (a) Amount of IF3<sub>mt</sub> (C+L) and IF3<sub>mt</sub>(C-L) bound to 28S subunits as a function of added protein. The amount of IF3<sub>mt</sub> or its derivatives bound to 28S subunits was determined by Microcon centrifugation followed by immunological detection using the standard curves for IF3<sub>mt</sub>(C+L) and IF3<sub>mt</sub>(C-L) (Supplementary Fig. S1). The apparent  $K_d$  values were estimated from the linear range of the calibration curves. (b) The amount of IF3<sub>mt</sub>(N+L) and IF3<sub>mt</sub>(N-L) bound to 28S subunits as a function of added protein was determined as above.

with and without the linker strongly binds to the small subunit, having only a two- and threefold lower affinity than full-length IF3<sub>mt</sub> for the 28S subunit.

In contrast to the N-domain of *E. coli* IF3, the N-domain of IF3<sub>mt</sub> binds to 28S subunits reasonably well, having only a 10-fold lower affinity than the full-length factor for the small subunit (Table 1). This result indicates that much of the ribosome binding interactions are mediated by the C-domain but that the N-domain clearly contributes to this interaction. These data also indicate that the linker region contributes only mildly to the tightness of the interaction between IF3<sub>mt</sub> and the small subunit. This observation is in contrast to observations made with chloroplast IF3 in which mutations of conserved lysine residues in the linker region had drastic effects (100-fold) on the binding of IF3<sub>chl</sub> to ribosomal subunits.<sup>20</sup>

The results obtained for the binding of IF3<sub>mt</sub> and the IF3<sub>mt</sub>(N+L) derivative to 28S subunits obtained by Microcon centrifugation were compared to those obtained by sucrose density gradient centrifugation. For these experiments, IF3<sub>mt</sub> and IF3<sub>mt</sub>(N+L) bound to the small subunits were separated from unbound protein by centrifugation (Supplementary Fig. S2). The  $K_d$  values obtained using the sucrose density gradient centrifugation method are 35 and 277 nM for IF3<sub>mt</sub> and IF3<sub>mt</sub>(N+L), respectively, which are very similar to those obtained using the Microcon method.

The experiments above were carried out on His-tagged derivatives of IF3<sub>mt</sub>. The interaction of IF3<sub>mt</sub> and its domains with 28S subunits could potentially be affected by the presence of the His-tag. To assess this possibility, we prepared and tested untagged derivatives of IF3<sub>mt</sub>, IF3<sub>mt</sub>(N+L), and IF3<sub>mt</sub>(C+L) for their activities in initiation complex formation on 55S ribosomes and for binding to 28S subunits (Supplementary Fig. S4). The untagged derivatives had essentially the same activities as the His-tagged variants in stimulating initiation complex formation. Further, the  $K_d$  values for the binding of untagged IF3<sub>mt</sub> and its domains to 28S subunits are 40, 70, and 232 nM for IF3<sub>mt</sub>, IF3<sub>mt</sub>(C+L), and IF3<sub>mt</sub>(N+L), respectively (Supplementary Fig. S4). These values are essentially the same as those obtained for the His-



tagged derivatives, indicating that the tag was not affecting the properties of IF3<sub>mt</sub> or its derivatives.

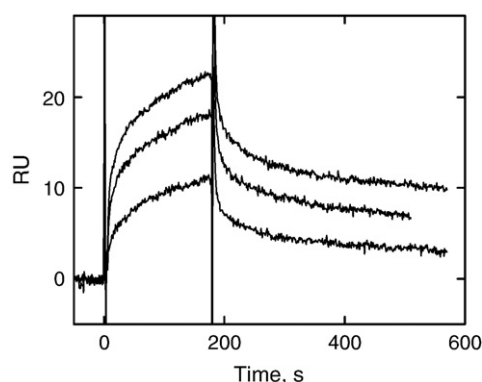
### Kinetics of the binding of IF3<sub>mt</sub> and its C-domain to 28S subunits using surface plasmon resonance

The rates of association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) of IF3<sub>mt</sub> and its C-domain from 28S subunits were determined by surface plasmon resonance (SPR). IF3<sub>mt</sub> and IF3<sub>mt</sub>(C+L) were active after the coupling reaction and were capable of binding to 28S subunits. However, the C-domain without the linker and the N-domain with or without the linker were inactive when coupled to the sensor chip, and their binding to 28S subunits could not be studied using this method.

The kinetics of the binding of IF3<sub>mt</sub>(C+L) at different concentrations of 28S subunits are shown in Fig. 4. The rate constants were determined as described previously,<sup>18</sup> and the values are shown in Table 2. The  $k_{on}$  of the C-domain of IF3<sub>mt</sub> is twofold slower than that of the full-length factor, while  $k_{off}$  is almost the same for both. This result indicates that the N-domain of IF3<sub>mt</sub> may play a small role in promoting the fast association of this factor with 28S subunits. The equilibrium dissociation constant was calculated from the ratio of  $k_{off}/k_{on}$ . These  $K_d$  values agree with the values obtained using the Microcon assay. This observation suggests that there are no hidden intermediates during formation of the IF3<sub>mt</sub>·28S or IF3<sub>mt</sub>(C+L)·28S complexes.

### Release of IF3<sub>mt</sub>(N+L) from 28S subunits in the presence of 39S subunits

The C-domain of IF3 is believed to bind to the platform of the small subunit in a region where there are significant contacts with the large subunit. Hence, it is logical that the C-domain would be displaced



**Fig. 4.** The forward and reverse rate constants for the binding of IF3<sub>mt</sub>(C+L) to 28S subunits determined by SPR. Sensorgram of IF3<sub>mt</sub>(C+L) binding to 28S subunits. The subunits were diluted to different concentrations (25, 50, and 75 nM), and 60  $\mu$ L of this dilution was injected onto CM5 chips containing immobilized IF3<sub>mt</sub>(C+L) or an avidin control at a flow rate of 20  $\mu$ L/min. The RU values shown are due to the binding of 28S subunits to IF3<sub>mt</sub>(C+L) [RU change due to IF3<sub>mt</sub>(C+L)–RU change due to avidin].

**Table 2.** Kinetic rate constants ( $k_{on}$  and  $k_{off}$ ) and calculated  $K_d$  values for the interaction of IF3<sub>mt</sub> and IF3<sub>mt</sub>(C+L) with the 28S subunit using SPR

Protein	$k_{on}$ ( $M^{-1} s^{-1}$ )	$k_{off}$ ( $s^{-1}$ )	$K_d$ (nM)
IF3 <sub>mt</sub> <sup>a</sup>	$(3.80 \pm 0.2) \times 10^5$	$(1.35 \pm 0.02) \times 10^{-2}$	$37 \pm 9$
IF3 <sub>mt</sub> (C+L)	$(2.10 \pm 0.1) \times 10^5$	$(0.92 \pm 0.1) \times 10^{-2}$	$44 \pm 8$

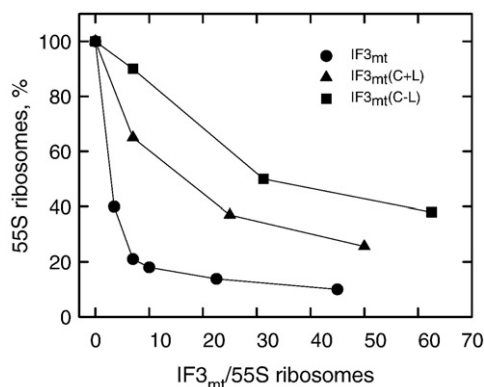
<sup>a</sup> Rate constants have been taken from previous results.<sup>18</sup>

by the large subunit. However, it is less clear where the N-domain is positioned on the small subunit and whether the large subunit would promote its dissociation. To investigate this question, IF3<sub>mt</sub>(N+L) was bound to the small subunit. The 39S subunit was subsequently added and the amount of IF3<sub>mt</sub>(N+L) remaining associated with the ribosome was determined. No detectable IF3<sub>mt</sub>(N+L) was present in the resulting 55S ribosomes (data not shown), indicating that the N-domain is displaced by the large subunit.

### Dissociation of 55S ribosomes by IF3<sub>mt</sub> and its N- and C-domain

To explore the role of the N- and C-domains and the linker region of IF3<sub>mt</sub> on the dissociation of mitochondrial 55S ribosomes, we incubated the ribosomes with different concentrations of IF3<sub>mt</sub> or its derivatives and we determined the distribution of ribosomal particles by sucrose density gradient centrifugation (Supplementary Fig. S3). In the absence of IF3<sub>mt</sub>, most of the ribosomes are present as 55S particles at 5 mM MgCl<sub>2</sub>. IF3<sub>mt</sub> had a significant effect on the fraction of the ribosomes that sedimented as 55S particles with about 50% dissociation observed at a ratio of 3 IF3<sub>mt</sub>/ribosome. The isolated N-domain with or without the linker region was inactive in promoting subunit dissociation even at very high concentrations (an IF3<sub>mt</sub> N-domain/55S ratio of 110:1). The C-domain with the linker had considerable activity in promoting subunit dissociation but was somewhat less active than the full-length factor. An 18-fold excess of IF3<sub>mt</sub>(C+L) over 55S ribosomes was required to achieve 50% dissociation of the particles. This construct has only a twofold weaker binding to 28S subunits compared to intact IF3<sub>mt</sub>. Hence, it is less effective at subunit dissociation than one would predict based on the strength of its interaction with the small subunit. One possible explanation for this observation is that the C-domain alone is more readily displaced by the 39S subunit. The intact factor has contacts with the 28S subunit through both the N- and C-domains, making it more difficult for the 39S subunit to displace it from the small subunit.

The linker region clearly contributes to the ability of the C-domain to promote subunit dissociation (Fig. 5). A ratio of more than 30 IF3<sub>mt</sub>(C–L) to ribosomes is required for 50% dissociation with this derivative. This value is also quite different from what one would expect based on the binding constant for the interaction of IF3<sub>mt</sub>(C–L) with 28S



**Fig. 5.** Effect of IF3<sub>mt</sub> and its C-domain with and without the linker on the dissociation of mitochondrial 55S ribosomes. Dissociation of 55S ribosomes (60 nM to 80 nM) was monitored in the presence of different protein concentrations of IF3<sub>mt</sub> or its C-domain derivatives. The concentrations of IF3<sub>mt</sub> were 0.18, 0.36, 0.54, 1.8, and 3.6  $\mu$ M. The concentrations of IF3<sub>mt</sub>(C+L) were 0.37, 2.0, and 4.0  $\mu$ M and the concentrations of IF3<sub>mt</sub>(C-L) were 0.37, 2.5, and 5.0  $\mu$ M, respectively. Representative fractionation profiles of mitochondrial 55S ribosome dissociation in the absence and presence of full-length IF3<sub>mt</sub> and its domains are shown in Supplementary Fig. S3. The area under the 55S peak in the absence of protein was set to 100% 55S ribosomes. The percentage of this value remaining was calculated in the presence of IF3<sub>mt</sub> or its derivatives and plotted as a function of the ratio of IF3<sub>mt</sub> to 55S ribosomes.

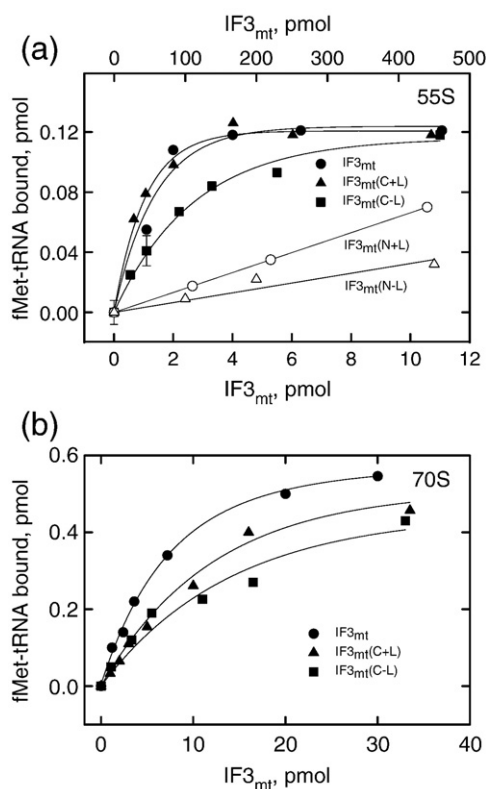
subunits, which is only about threefold weaker than that of the intact factor. This observation again suggests that the linker region makes it more difficult for the 39S subunit to displace IF3<sub>mt</sub> from the 28S subunit and its removal leads to a more facile displacement of the C-domain by the large subunit.

#### Effect of the N- and C-domains of IF3<sub>mt</sub> on initiation complex formation

The activities of the IF3<sub>mt</sub> and its N- and C-domains were examined by measuring their effect on fMet-tRNA binding to mitochondrial 55S and *E. coli* 70S ribosomes (Fig. 6) in the presence of IF2<sub>mt</sub> and poly(A,U,G). IF3<sub>mt</sub>(C+L) is as active as full-length IF3<sub>mt</sub> in promoting initiation complex formation on 55S ribosomes. This assay is believed to measure primarily the dissociation factor activity of IF3<sub>mt</sub>. IF3<sub>mt</sub>(C+L) is somewhat less active than the full-length factor in promoting ribosome dissociation as measured by sucrose density gradient centrifugation. In the centrifugation assay, the apparent degree of dissociation reflects a competition between IF3<sub>mt</sub> binding and the reassociation of the 28S subunit with the 39S subunit, leading to an apparent decrease in ribosome dissociation. In contrast, in the fMet-tRNA binding assay, the displacement of IF3<sub>mt</sub> by the 39S subunit leads to the 55S initiation complex, which is measured in the assay. Thus, the high activity of IF3<sub>mt</sub>(C+L) in the fMet-tRNA binding assay most likely reflects

its significant activity in subunit dissociation. IF3<sub>mt</sub>(C-L) is somewhat less active (about 30–40%) in the fMet-tRNA binding assay. This reduced level of activity is likely to reflect the significantly lower activity of this construct in subunit dissociation. These observations suggest that the C-domain including the linker region is able to carry out the most basic function of IF3<sub>mt</sub> of promoting initiation complex formation beginning with 55S ribosomes. Basically, similar results were obtained when *E. coli* 70S ribosomes were used in this assay in place of mitochondrial ribosomes (Fig. 6b).

To test possible effects of the N-domain on fMet-tRNA binding to ribosomes, it was important to use significantly higher concentrations of the derivatives due to the lower binding constants observed with

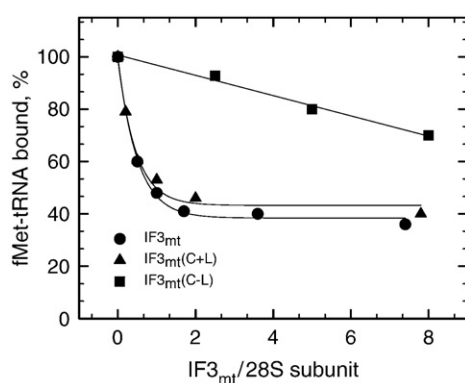


**Fig. 6.** Effect of IF3<sub>mt</sub> and its C- and N-domains with and without the linker on initiation complex formation. (a) [<sup>35</sup>S]fMet-tRNA binding to mitochondrial 55S particles (5 pmol, 50 nM) was tested in the presence of different concentrations of IF3<sub>mt</sub> and its C- and N-domain derivatives at a fixed amount of IF2<sub>mt</sub> (100 nM) using 10  $\mu$ g poly(A,U,G) as the mRNA. A blank (about 0.18 pmol) representing the amount of label retained on the filter in the absence of IF3<sub>mt</sub> but in the presence of IF2<sub>mt</sub> has been subtracted from each value. The lower x-axis corresponds to full-length IF3<sub>mt</sub> and its C-domain derivatives, and the upper x-axis corresponds to the N-domain derivatives. (b) [<sup>35</sup>S]fMet-tRNA binding to *E. coli* 70S particles (20 pmol, 200 nM) was tested in the presence of different amounts of IF3<sub>mt</sub> and its C-domain derivatives at a fixed concentration of IF2<sub>mt</sub> (100 nM) using poly(A,U,G) as the mRNA. A blank representing the amount of label retained on the filter in the absence of IF3<sub>mt</sub> has been subtracted from each value (0.54 pmol). Representative errors are shown in (a).

them (Table 1). IF3<sub>mt</sub>(N+L) stimulates the binding of fMet-tRNA to 55S ribosomes in the presence of IF2<sub>mt</sub> slightly (Fig. 6). This stimulation is enhanced about twofold by the presence of the linker in agreement with the higher activity of IF3<sub>mt</sub>(C+L) compared to IF3<sub>mt</sub>(C-L). The degree of stimulation observed with the N-domain derivatives is much lower than would be predicted from the reduction in the binding constant alone. It is in agreement with the undetectable ribosome dissociation activities of these derivatives (Supplementary Fig. S3). The slight increase in fMet-tRNA binding observed in the presence of the N-domain derivatives is intriguing. It may reflect an ability of the N-domain to enhance the reassociation of the 39S subunit stabilizing transient complexes formed or perhaps an enhancement of the binding of IF2<sub>mt</sub> to the small subunit.

#### Effect of IF3<sub>mt</sub> and its N- and C-domain on the binding of fMet-tRNA to 28S subunits in the absence of mRNA

In previous studies,<sup>17</sup> it has been shown that the C-terminal extension on IF3<sub>mt</sub> plays an important role in reducing fMet-tRNA binding to 28S subunits in the absence of mRNA. It has no effect on fMet-tRNA binding to the small subunit in the presence of mRNA. The C-domain with the linker and that without the linker were tested for this effect (Fig. 7). IF3<sub>mt</sub>(C+L) was as effective as the full-length factor in reducing fMet-tRNA binding in the absence of mRNA. This effect is strongly influenced by the presence of the linker region since IF3<sub>mt</sub>(C-L) had little effect in this assay. These results, taken together with previous observations, suggest that both the C-terminal extension and the linker are important for this activity. The C-terminal extension is predicted to emerge from the C-terminal domain pointing toward the linker region in agreement with this idea.<sup>17</sup>



**Fig. 7.** Effect of IF3<sub>mt</sub> derivatives on the binding of [<sup>35</sup>S] fMet-tRNA to mitochondrial 28S subunits in the absence of mRNA. (a) Reaction mixtures contained 160 nM IF2<sub>mt</sub> and increasing levels of IF3<sub>mt</sub> or its C-domain derivatives at a constant level of 28S subunits (50 nM). In the absence of IF3<sub>mt</sub>, 0.1 pmol of fMet-tRNA was bound to the 28S subunits by IF2<sub>mt</sub> in the reaction mixture. The estimated error of this experiment is  $\pm 10\%$ .

The effects of IF3<sub>mt</sub>(N+L) and IF3<sub>mt</sub>(N-L) on the IF2<sub>mt</sub>-dependent binding of fMet-tRNA to 28S subunits were also tested in the absence and presence of poly(A,U,G) or the AUG triplet. For these experiments, higher levels of IF3<sub>mt</sub>(N+L) and IF3<sub>mt</sub>(N-L) were used, reflecting their lower binding constants for the small subunit. The N-domain, both with or without the linker, had no effect on fMet-tRNA binding to the small subunit in the absence of mRNA and showed a very slight ( $\sim 10\%$ ) stimulation in the presence of the AUG triplet (data not shown).

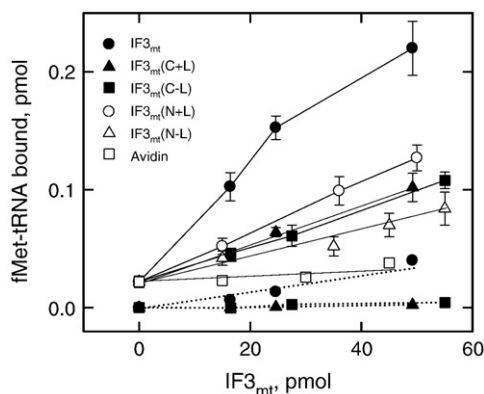
#### Effect of IF3<sub>mt</sub> and its domains on the formation of a complex between [<sup>35</sup>S]fMet-tRNA and IF2<sub>mt</sub>

Previous work<sup>21</sup> has shown that IF2<sub>mt</sub> like *E. coli* IF2, can form a binary complex in solution with fMet-tRNA mediated primarily through the C-terminal domain of IF2. The  $K_d$  for the binding of fMet-tRNA to IF2<sub>mt</sub> is about 1  $\mu$ M.<sup>21</sup> The interaction between IF2<sub>mt</sub> and mitochondrial fMet-tRNA is specific and is 50-fold stronger than the binding of the unformylated mitochondrial initiator tRNA, Met-tRNA. Bovine IF2<sub>mt</sub> also has a high affinity for yeast cytoplasmic fMet-tRNA and *E. coli* fMet-tRNA.<sup>22</sup> The effect of IF3<sub>mt</sub> on the interaction between IF2<sub>mt</sub> and fMet-tRNA was examined using a filter binding assay (Fig. 8). Interestingly, IF3<sub>mt</sub> stimulated the formation of this complex nearly 10-fold, perhaps promoting the formation of a ternary complex containing IF3<sub>mt</sub>:IF2<sub>mt</sub>:fMet-tRNA. Both the N- and C-domains of IF3<sub>mt</sub> are involved in the stimulation of fMet-tRNA binding to IF2<sub>mt</sub> (Fig. 8), with the N-domain being somewhat more effective than the C-domain in this interaction. The effect of IF3<sub>mt</sub> on the interaction between IF2<sub>mt</sub> and Met-tRNA was also examined (data not shown). IF3<sub>mt</sub> has a very minor effect on IF2<sub>mt</sub>:Met-tRNA complex formation. This result indicates that the stimulation of fMet-tRNA binding to IF2<sub>mt</sub> by IF3<sub>mt</sub> is largely specific for the formylated initiator tRNA.

Since IF3<sub>mt</sub> is a basic protein ( $pI=10.8$ ), it was essential to ensure that the effect observed was not simply due to an electrostatic interaction between IF3<sub>mt</sub> and IF2<sub>mt</sub> ( $pI \sim 6$ ) or the acidic fMet-tRNA. Two controls were carried out. In the first, IF3<sub>mt</sub> alone was incubated with fMet-tRNA and its ability to retain the tRNA on the filter was tested. Like *E. coli* IF3,<sup>23,24</sup> IF3<sub>mt</sub> had a measurable affinity for RNA in general. However, this binding was small compared to the stimulation of IF2<sub>mt</sub>:fMet-tRNA complex formation at the IF3<sub>mt</sub> concentrations used in the current experiments. None of the domain constructs had significant tRNA binding activity (Fig. 8).

The second control was to test the effect of a nonspecific basic protein on the formation of the IF2<sub>mt</sub>:fMet-tRNA complex. Neither avidin ( $pI=10.5$ ) nor lysozyme ( $pI=10.7$ ) had a significant effect on the formation of the IF2<sub>mt</sub>:fMet-tRNA complex, indicating that the results obtained with IF3<sub>mt</sub> reflect a specific interaction between IF2<sub>mt</sub> and





**Fig. 8.** Effect of IF3<sub>mt</sub> and its domains on the binding of [<sup>35</sup>S]fMet-tRNA to IF2<sub>mt</sub>. [<sup>35</sup>S]fMet-tRNA binding to IF2<sub>mt</sub> (17 pmol, 170 nM) was tested in the presence of different concentrations of IF3<sub>mt</sub> and its C- and N-domain derivatives. The continuous lines represent the binding observed in the presence of IF2<sub>mt</sub> alone or in the presence of either IF3<sub>mt</sub> or its domains. The broken lines represent the amount of label retained on the filter in the presence of IF3<sub>mt</sub> or its derivatives alone. This value has been subtracted from the data plotted in the presence of IF2<sub>mt</sub> to provide a clear estimate of the stimulatory effect of IF3<sub>mt</sub> on the interaction of IF2<sub>mt</sub> with fMet-tRNA.

IF3<sub>mt</sub>. It is conceivable that this interaction takes place in solution prior to initiation factor binding to the small subunit. Alternatively, this interaction may reflect contacts between these factors on the surface of the 28S subunit.

## Discussion

The IF3 from both bacteria and mitochondria is folded into distinct N- and C-domains separated by a linker of approximately 25 residues. Previous work<sup>9</sup> with *E. coli* IF3 indicates that the C-domain binds independently to the small ribosomal subunit and that this domain can carry out all the known *in vitro* activities of this factor. However, this domain binds 30S subunits with a 100-fold lower affinity than the intact factor. This result is in sharp contrast to those observed with IF3<sub>mt</sub>. The C-domain with the linker region of the human mitochondrial factor has a strong affinity for mitochondrial 28S subunits having a  $K_d$  only about twofold weaker than the full-length factor. The C-domain of *E. coli* IF3 is believed to bind to the platform region of the small subunit, physically blocking interaction with the large subunit.<sup>7</sup> It makes contacts with helices H23, H24, and H45, blocking the formation of intersubunit bridges B2b, B2c, and B7a. Examination of the truncated small subunit ribosomal RNA (12S) of mitochondrial ribosomes indicates that these helices have been preserved and that intersubunit bridges B2b, B2c, and B7a represent three out of six conserved bridges between the subunits of the mitochondrial ribosome.<sup>2</sup>

It is not immediately apparent why the independent C-domain of IF3<sub>mt</sub> binds so much better to the

small subunit than does the bacterial factor. The C-domain of IF3<sub>mt</sub> does have an extension of about 30 amino acids following the region with homology to the bacterial factors. However, deletion of this region does not affect the binding of IF3<sub>mt</sub> to the 28S subunit.<sup>18</sup> Rather, the C-terminal extension is thought to play an important role in promoting the dissociation of IF3<sub>mt</sub> from 28S subunits upon 39S subunit joining.<sup>18</sup> The platform region of the small subunit where the C-domain binds is one of the more highly conserved regions between bacterial and mitochondrial ribosomes.<sup>2,25</sup> However, the edges of the platform do contain proteins that are specific to the mitochondrial ribosome, and it is possible that IF3<sub>mt</sub> makes contacts with these proteins that are unavailable to the bacterial factor. It should also be noted that different experimental strategies were used in the *E. coli* and mitochondrial systems to determine the strength of the interaction. The binding of the C-domain of IF3<sub>mt</sub> was measured directly and independently while that of the *E. coli* factor was determined in competition with the full-length factor.<sup>9</sup>

In *E. coli* IF3, the linker does not appear to contribute to the binding of this factor to 30S subunits or to have any functional activity other than to provide a physical connection between the N- and C-terminal domains.<sup>9</sup> In contrast, the linker in IF3<sub>mt</sub> plays a functional role in binding to the small subunit, in the dissociation of 55S ribosomes, and in initiation complex formation (Figs. 5 and 6). This observation suggests that the linker has direct and important contacts with the surface of the small subunit and is not simply a physical connector between the two domains.

In the bacterial system, no binding of the independent N-domain to 30S subunits was detected,<sup>9</sup> but NMR data indicate that this domain does interact with 30S subunits through a small number of residues in the full-length factor.<sup>26</sup> Again, this observation differs from that made with the N-domain of IF3<sub>mt</sub>, which binds to the 28S subunit with only a 10-fold lower affinity than the full-length factor. There are considerable differences of opinion about the location of the binding site for the N-domain of IF3.<sup>7,13</sup> One recent model suggests that this region of IF3 is located near A790 in helix 24 close to the P-site tRNA. This position places the N-domain close to the neck at the border between the platform and the body of the small subunit.<sup>13</sup> This position is in agreement with the cross-linking of IF3 to ribosomal proteins S11, S13, and S19. While mammalian mitochondrial ribosomes have a homolog of S11, they do not have homologs of either S13 or S19 in the head of the small subunit. Rather, the head of the 28S subunit has several unidentified proteins specific to the mitochondrial ribosome. Interactions between the N-domain of IF3<sub>mt</sub> and these proteins could explain the significant binding of the N-domain to the small subunit observed with the mitochondrial factor.

One interesting suggestion for the role of the N-domain of IF3 proposed by Fabbretti *et al.* is that a



fluctuating interaction between the N-domain and the neck of the 30S subunit modulates the association and dissociation of the factor from the subunit.<sup>13</sup> The significant binding observed with both the C-domain and the isolated N-domain of IF3<sub>mt</sub> argues that, in the mitochondrial system, this role may not be the major function of the N-domain. Kinetic analysis indicates that  $k_{on}$  is reduced only about twofold when the N-domain is removed while the  $k_{off}$  value is nearly unchanged. Thus, unlike *E. coli* IF3, the N-domain of IF3<sub>mt</sub> itself does not appear to have a strong role in modulating the interaction of the C-domain with the 28S subunit.

The role of the N-domain remains obscure. However, one possible hint for its effect lies in the stimulatory effect of IF3<sub>mt</sub> on the formation of the complex between IF2<sub>mt</sub> and fMet-tRNA. Both the N- and C-domains appear to play a role in this interaction. While this stimulation has been observed in solution, it may reflect an interaction that occurs on the surface of the small subunit in the 28S initiation complex. It should be noted that the N-domain has been positioned near A790<sup>13</sup> and that this residue interacts with the anticodon stem-loop of the P-site-bound initiator tRNA.<sup>27</sup>

Previous data also suggest that IF3 is positioned close to the P-site-bound fMet-tRNA on the small subunit.<sup>7</sup> The C-domain of IF3 is located close to the junction of the D-stem and the anticodon stem and is close to the anticodon itself. The N-domain including the linker has been positioned close to the acceptor stem and the D-arm on the opposite side of the tRNA seen with the C-domain. These data are quite compatible with the observations made here. Our data indicate that the C-domain helps promote the formation of the complex between IF2<sub>mt</sub> and that the N-domain, particularly with the linker, also facilitates the formation of this complex.

As indicated above, it seems unlikely that a major role of the N-domain of IF3<sub>mt</sub> is to modulate an interaction with the small subunit as suggested for the bacterial factor. Rather, it seems more likely that this region of IF3<sub>mt</sub> plays a role in promoting conformational changes in the small subunit prior to fMet-tRNA binding and that it may have a role in the kinetics of initiation complex formation. Since the majority of the mRNAs of mammalian mitochondria do not contain a 5' untranslated leader region, it is also possible that the N-domain of IF3<sub>mt</sub> may play a role in ensuring the correct positioning of the 5' start codon in the P-site of the ribosome. This possibility is currently under investigation.

## Materials and Methods

### Materials

High purity grade chemicals were purchased from Sigma-Aldrich or Fisher Scientific. A rabbit polyclonal primary antibody to the region of IF3<sub>mt</sub> homologous to the bacterial factors was made by Pacific Immunology Corporation. Secondary antibody, goat anti-rabbit immu-

noglobulin G coupled to alkaline phosphatase, was purchased from Sigma-Aldrich. Protein-free blocking agent was purchased from Pierce Technologies. Microcon-100 spin columns and pure nitrocellulose membrane filters were obtained from Millipore Corporation. Research-grade CM5 sensor chips, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, N-hydroxysuccinimide, and 2-(2-pyridinyldithio)-ethaneamine hydrochloride were obtained from Biacore Company. Bovine mitochondria and mitochondrial ribosomes (55S), ribosomal subunits (28S and 39S), bovine IF2<sub>mt</sub>, yeast [<sup>35</sup>S]Met-tRNA, and yeast [<sup>35</sup>S]fMet-tRNA were prepared as described previously.<sup>28–30</sup>

### Cloning of C- and N-domains of IF3<sub>mt</sub>

The C-domain with the linker and that without the linker were amplified by PCR using the mature IF3<sub>mt</sub> cDNA as template,<sup>16</sup> using forward primers GGAATTC-CATATGACAGGATTGCAGAT (for the C-domain with the linker) and GGAATTGCCATATGAGAAAGGAAC-TGATTTTG (for the C-domain without the linker) and the reverse primer CCTTAACTCGAGCTGATGCAGAAC (for both). The N-domain with the linker and that without the linker were prepared using the forward primer GGAATTCATATGACAGCACCAGCACAG (for both) and the reverse primers CCTTAACTCGAGCAGGGTTGGTTTC-CAGTTTTGGG (with the linker) and CCTTAACTCGAGCTGATACTCTGCAGGTTC (without the linker). These PCR products were purified and digested with NdeI and XhoI and cloned into the pET21 (c+) vector (Novagen), providing a six His-tag at the C-terminus of the expressed protein. Constructs carrying the IF3<sub>mt</sub> domains were transformed into *E. coli* DH5α, and the nucleotide sequence of the inserted DNA was confirmed. The plasmids were subsequently transformed into *E. coli* BL21pArgU218.<sup>16</sup>

For removal of the His-tag, IF3<sub>mt</sub> and its C- and N-domains with the linker were prepared carrying a tobacco etch virus (TEV) cleavage site between an N-terminal His-tag and the desired region of IF3<sub>mt</sub>. For these constructs, PCR was used to amplify the mature IF3<sub>mt</sub> cDNA template<sup>16</sup> with forward primers GGAATTCATATGGAAAACCTGTA-TTTTCAGGGAACAGCACCAGCACAG (for full-length and N-domain IF3<sub>mt</sub>) and GGAATTCATATGGAAAA-CCTGTATTTTCAGGGAATGACAGGATTGCAGATC (for the C-domain) and reverse primers CCTTAACTCGAGT-TATTACTGATGCAGAAC (for full-length and C-domain) and CCTTAACTCGAGTTATTACAGGGTTGGTC-CAGTTTTGGG (for the N-domain). These PCR products were purified, digested with NdeI and XhoI, and cloned into pET15b (Novagen), providing a six His-tag at the N-terminus followed by a TEV cleavage site preceding the region of IF3<sub>mt</sub> to be studied. Constructs carrying IF3<sub>mt</sub> and its domains were transformed into XL10GOLD *E. coli* DH5α, and the nucleotide sequence of the inserted DNA was confirmed. The plasmids were subsequently transformed into *E. coli* BL21RIL for expression.

### Expression and purification of IF3<sub>mt</sub> and its C- and N-domain derivatives with and without the linker

The purification of the mature form of IF3<sub>mt</sub> and its domains was carried out as described previously<sup>16,17</sup> using Ni-NTA resins. IF3<sub>mt</sub>(C+L) and IF3<sub>mt</sub>(C–L) did not require further purification. However, the Ni-NTA preparations of IF3<sub>mt</sub>, IF3<sub>mt</sub>(N+L), and IF3<sub>mt</sub>(N–L) contained degradation products and were further purified by HPLC using a TSK-

gel SP5-PW column.<sup>31</sup> These derivatives expressed well although cells carrying the IF3<sub>mt</sub>(C–L) plasmid were slow to emerge from stationary phase.

The expressed N-terminally His-tagged proteins containing the TEV cleavage site were purified using Ni-NTA resins and did not require further purification. Proteins were digested with pro-TEV protease (Promega) in which the protease itself contains a His-tag following the manufacturer's instructions. The protease and any uncut IF3<sub>mt</sub> remaining were removed using Ni-NTA resins. Untagged IF3<sub>mt</sub>, IF3<sub>mt</sub>(C+L), and IF3<sub>mt</sub>(N+L) protein were used to confirm the idea that the His-tag does not affect the interaction of IF3<sub>mt</sub> or its derivatives with 28S subunits.

#### Estimation of the exposure of IF3<sub>mt</sub> and its derivatives to antibodies when bound to 28S subunits

Reaction mixtures (100  $\mu$ L) contained Binding Buffer (10 mM Tris–HCl, pH7.6, 7.5 mM MgCl<sub>2</sub>, 40 mM KCl, 0.1 mM spermine, and 1 mM dithiothreitol) with and without 28S subunits (5 pmol) with different amounts of IF3<sub>mt</sub> (0.16 to 0.66 pmol) and its derivatives [0.82 to 2.5 pmol for IF3<sub>mt</sub>(C+L) and IF3<sub>mt</sub>(N+L) and 2 to 6.5 pmol for IF3<sub>mt</sub>(C–L) and IF3<sub>mt</sub>(N–L)]. The samples were incubated for 10 min at 25 °C. The reaction mixtures were applied to the dot blot apparatus, and the IF3<sub>mt</sub> epitopes exposed to antibody in the absence and presence of 28S subunits were quantified colorimetrically as described previously.<sup>18</sup>

#### Quantitation of the binding of IF3<sub>mt</sub> and its C- and N-domain to mitochondrial 28S subunits using Microcon centrifugation

The binding of IF3<sub>mt</sub> and its derivatives to 28S subunits was tested by Microcon centrifugation as described previously<sup>18</sup> using 50 nM 28S subunits (5 pmol) and different concentrations of IF3<sub>mt</sub> or its domain derivatives as indicated. The ribosome:protein mixtures were incubated for 20 min at 25 °C. Unbound proteins were separated from protein bound to 28S subunits using a Microcon spin filter device as described previously.<sup>18</sup> The bound IF3<sub>mt</sub> and its derivatives were applied to a dot blot apparatus, and the binding of IF3<sub>mt</sub> to 28S subunits was quantified colorimetrically using antibodies raised against IF3<sub>mt</sub>. As expected, the presence of 28S subunits quenched the antibody–antigen response in the dot blot. This quenching effect was fully overcome by adding EDTA to a final concentration of 20 mM prior to carrying out the dot blot. The amount of bound protein was determined from individual calibration curves generated for each construct separately to account for the different antigenicity of the derivatives (Supplementary Fig. S1).

It was necessary to determine the percentage of active molecules to estimate the apparent equilibrium dissociation constant for the interaction of IF3<sub>mt</sub> and its domain derivatives with 28S subunits. These values were determined as described previously<sup>32</sup> and indicated that the IF3<sub>mt</sub> and its derivatives were almost 100% active while the 28S subunits were 25–50% active.

#### Binding of the N-domain of IF3<sub>mt</sub> to 28S subunits and its release in the presence of 39S subunits

The binding of IF3<sub>mt</sub>(N+L) to 28S subunits was performed at a fixed concentration of ribosomes (50 nM) and different concentrations of protein (2.2, 4.4, and

6.6 nM). The ribosomes alone and ribosome:protein mixtures were incubated for 20 min at 25 °C in Binding Buffer (100  $\mu$ L). Subsequently, 39S subunits (6.6 nM) were added to each reaction mixture (giving a final MgCl<sub>2</sub> concentration of 8 mM) and incubated for an additional 5 min. This concentration of Mg<sup>2+</sup> is sufficient to promote quantitative association of the subunits to 55S particles. Unbound proteins were separated from protein bound to 55S ribosomes using a Microcon spin filter device as described previously.<sup>18</sup> EDTA (20 mM) was added to samples containing ribosomes, and the amount of IF3<sub>mt</sub>(N+L) associated with the ribosomes was determined using the dot blot.

#### Binding of IF3<sub>mt</sub> IF3<sub>mt</sub>(C+L) to 28S subunits using SPR

The rate constants governing the association and dissociation of IF3<sub>mt</sub> and IF3<sub>mt</sub>(C+L) with 28S subunits were measured using a Biacore 2000 biosensor instrument located in the University of North Carolina Macromolecular Interactions Facility as described previously.<sup>18</sup> The extent of immobilization was kept low [ $\sim$ 200 resonance units (RU)] to avoid crowding of the surface. Samples (200  $\mu$ L) of different concentrations of 28S subunits (25, 50, and 75 nM) were prepared in Running Buffer (20 mM Hepes–KOH, pH7.6, 7.5 mM MgCl<sub>2</sub>, 100 mM KCl, 1 mM DTT, 0.5% glycerol, and 0.01% surfactant P20), and 60  $\mu$ L of each solution was injected at a flow rate of 20  $\mu$ L/min. The RU change due to the binding of 28S subunits was obtained by subtracting the RU of the control surface (avidin bound at 200 RU) from the RU obtained from the surface containing IF3<sub>mt</sub> or IF3<sub>mt</sub>(C+L). Of the IF3<sub>mt</sub> derivatives, only IF3<sub>mt</sub>(C+L) retained activity when coupled to the surface. Rate constants for association and dissociation were calculated based on the approach outlined previously.<sup>18,33</sup>

#### Dissociation of mitochondrial 55S ribosomes by IF3<sub>mt</sub> and its N- and C-domains

Mitochondrial ribosomes (60 nM) were incubated in the presence or absence of IF3<sub>mt</sub> or its domain derivatives at different concentrations as indicated in 100  $\mu$ L of Gradient Buffer (25 mM Tris–HCl, pH7.6, 5 mM MgCl<sub>2</sub>, 40 mM KCl, and 1 mM DTT) for 15 min at 37 °C. After incubation, reaction mixtures were placed on ice for 10 min and then layered onto a cold 4.8-mL 10–30% linear sucrose gradient prepared in Gradient Buffer. Gradients were centrifuged for 1 h 45 min in a Beckman SW55 Ti rotor and fractionated using an ISCO gradient fractionator.

#### Initiation complex formation on mitochondrial 55S and *E. coli* 70S ribosomes

Stimulation of [<sup>35</sup>S]fMet–tRNA binding to either mitochondrial or *E. coli* ribosomes in the presence of IF3<sub>mt</sub> or its C- and N-domain derivatives was examined using filter binding assays as described previously.<sup>16,17</sup> The amount of label retained on the filter in the absence of IF3<sub>mt</sub> has been subtracted from each value as indicated in the figure legends. Since high concentrations of IF3<sub>mt</sub>(N+L) and IF3<sub>mt</sub>(N–L) were used in several experiments, additional control experiments were performed to measure the retention of [<sup>35</sup>S]fMet–tRNA on the filter in the presence of IF2<sub>mt</sub> alone (no ribosomes added) with different concentrations of IF3<sub>mt</sub>(N+L) and IF3<sub>mt</sub>(N–L). The amount of [<sup>35</sup>S]fMet–tRNA bound on the filter due to

IF3<sub>mt</sub>(N+L) or IF3<sub>mt</sub>(N–L) alone (0.18 pmol) has been subtracted from appropriate values.

#### Effect of IF3<sub>mt</sub> on the binding of fMet–tRNA to IF2<sub>mt</sub>

[<sup>35</sup>S]fMet–tRNA binding to IF2<sub>mt</sub> in the presence of IF3<sub>mt</sub> in solution was determined using the filter binding assay. Reaction mixtures (100 µL) contained IF2<sub>mt</sub> (17 pmol, 0.17 µM), [<sup>35</sup>S]fMet–tRNA (60 nM), 50 mM Tris–HCl, pH 7.6, 5 mM MgCl<sub>2</sub>, 40 mM KCl, 1 mM DTT, 0.1 mM spermine, 0.25 mM GTP, 1.25 mM phosphoenolpyruvate, 0.9 U pyruvate kinase, and variable amounts of IF3<sub>mt</sub> or its N- and C-domains as indicated. The reactions were incubated for 10 min, diluted with ice-cold buffer [50 mM Tris–HCl, pH 7.6, 5 mM MgCl<sub>2</sub>, and 40 mM KCl], and filtered through nitrocellulose membranes with two buffer washes. The filters were dried for 8 min at 100 °C. The amount of [<sup>35</sup>S]fMet–tRNA retained as a complex with IF2<sub>mt</sub> and IF3<sub>mt</sub> was determined by scintillation counting.

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#### Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2008.09.077](https://doi.org/10.1016/j.jmb.2008.09.077)

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