# Initiator tRNA Binding by e/aIF5B, the Eukaryotic/Archaeal Homologue of Bacterial Initiation Factor IF2[†]

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ABSTRACT: To carry initiator Met-tRNA$_i^{Met}$ to the small ribosomal subunit, eukaryal and archaeal cells use a heterotrimeric factor called e/aIF2. These cells also possess a homologue of bacterial IF2 called e/aIF5B. Several results indicate that the mode of action of e/aIF5B resembles some function of bacterial IF2. The e/aIF5B factor promotes the joining of ribosomal subunits. Moreover, there is genetic evidence that the factor participates in the binding of initiator tRNA to the small ribosomal subunit. However, up to now, an interaction between e/aIF5B and initiator tRNA was not evidenced. In this study, we use an assay based on protection of aminoacyl-tRNA against spontaneous deacylation to demonstrate that archaeal aIF5B indeed can interact with initiator tRNA. In complex formation, aIF5B shows specificity toward the methionyl moiety of the ligand. The complex between *Saccharomyces cerevisiae* eIF5B and methionylated initiator tRNA is less stable than that formed with aIF5B. In addition, this complex is almost indifferent to the side chain of the esterified amino acid. These results support the idea that, beyond the channeling of Met-tRNA$_i^{Met}$ to the 40S subunit by e/aIF2, e/aIF5B comes to interact with initiator tRNA on the ribosome. Recognition of an aminoacylated tRNA species at this site would then allow translation to begin. In the case of archaea, this checkpoint would also include the verification of the presence of a methionine at the P site.

Initiation factors for translation in eukarya and archaea are distinct from those of bacteria (*1*−*4*). However, two initiation factors, e/aIF1A/IF1 and e/aIF5B/IF2, are universally conserved in all living cells (*5*). In bacteria, IF2 stimulates the binding of formyl-methionyl-initiator tRNA$_f^{Met}$ (fMet-tRNA$_f^{Met}$) to initiating 30S ribosomal subunits. Actually, bacterial IF2 is able to form a binary complex with fMet-tRNA$_f^{Met}$. Recognition of the initiator tRNA is mainly based on the presence of an N-blocked methionine esterified to tRNA (*6*−*9*). However, IF2 also recognizes the absence of strong base pairing between positions 1 and 72 at the end of the acceptor stem of the tRNA (*9*). In contrast to other tRNA carriers such as elongation factor EF1A, the binding of tRNA by IF2 does not depend on the nucleotidic state of the factor, free, bound to GTP, or bound to GDP. On the side of the factor, the C-terminal $\beta$-barrel domain has been clearly shown to bind fMet-tRNA$_f^{Met}$ with the same affinity and specificity as full-length IF2 (*10*−*12*).

After recruitment of the initiator tRNA and verification of the positioning of the initiating small ribosomal subunit on the start codon, bacterial IF2 promotes the joining of the 50S ribosomal subunit to the 30S subunit (*13*). To achieve this role, the GTP form of the factor is required. In the subsequent step, hydrolysis of GTP allows dissociation of the factor from the 70S ribosome and protein synthesis starts. A recent cryoelectron microscopy structure of the *Escherichia coli* translation initiation complex shows how IF2 bridges the two ribosomal subunits while its C-terminal $\beta$-barrel domain interacts with the 3′-end of initiator tRNA (*14*). As a consequence, initiator tRNA is forced into a novel orientation at the P site, and ribosome-bound IF2 adopts a structure different from the crystallographic structure of its free aIF5B homologue (*15*).

In eukarya and archaea, initiator Met-tRNA$_i^{Met}$ is carried toward the ribosome by a heterotrimeric e/aIF2 factor. The existence of this factor, which has no orthologue in bacteria, raises the question of the utility of the e/aIF5B factor. However, several lines of evidence indicate that e/aIF5B plays a role similar to that of IF2 in initiation of translation (*16*). In particular, GTP-bound eIF5B promotes the joining of ribosomal subunits (*17*, *18*), and GTP hydrolysis is required for release of eIF5B from the 80S ribosome (*19*, *20*). Further, genetic evidence supports the idea that eIF5B facilitates the binding of initiator tRNA to the small ribosomal subunit (*21*).

Up to now, comparison of the mechanisms of action of IF2 and of e/aIF5B has been limited by the fact that binding of initiator tRNA to e/aIF5B could not be detected (*15*). Here, by using an assay based on protection against deacylation, we succeeded in showing the formation of a complex between methionylated initiator tRNA and aIF5B from *Sulfolobus solfataricus* or *Pyrococcus abyssi*. Complex formation was also obtained with N-terminally truncated eIF5B from yeast. In all cases, the strength of the binding of tRNA does not vary whether GTP or GDP is present.

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With archaeal aIF5B, complex formation includes recognition of the methionyl moiety esterified to the tRNA. The sequence of the tRNA has no influence. The stability of the complex between *Saccharomyces cerevisiae* eIF5B and methionylated initiator tRNA is weaker than that of the complexes obtained with the archaeal factors. Poor recognition of the esterified methionine by the yeast factor accounts for this behavior.

## EXPERIMENTAL PROCEDURES

### *Gene Cloning and Expression*

*ss-aIF5B.*[1] The gene encoding ss-aIF5B was PCR amplified from *S. solfataricus* total DNA (*22*), and cloned between the *Nde*I and *Sac*II sites of pET3alpa (*23*) to give pET3a5Bss. The expression vector pET3alpa was derived from pET3a (Novagen) by the insertion between the *Nde*I and *Bam*HI sites of a synthetic polylinker (TATGCCCGGGCTCGAGGGTACCCCGCGGGCGGCCGCGTCGACC) containing restriction sites for *Sma*I, *Xho*I, *Kpn*I, *Sac*II, *Not*I, and *Sal*I.

Rosetta pLacI-RARE cells (Novagen) carrying pET3a5Bss were grown at 37 °C in 1 L of 2× TY medium containing ampicillin (50 $\mu$g/mL) and chloramphenicol (34 $\mu$g/mL). Expression was induced by adding IPTG to a final concentration of 1 mM when $OD_{650}$ reached a value of 1.6. The culture was then continued for 5 h at room temperature. Cells were disrupted by sonication in 40 mL of buffer A [10 mM MOPS (pH 6.7), 10 mM 2-mercaptoethanol, and 0.1 mM PMSF] supplemented with 200 mM NaCl. After centrifugation, the supernatant was heated for 10 min at 70 °C. Precipitated material was removed by centrifugation. The supernatant was treated with 1% (w/v) streptomycine sulfate and the resulting precipitate removed by centrifugation. Finally, the extract was concentrated by precipitation with ammonium sulfate to 80% saturation. The pellet was dissolved in 4 mL of buffer A supplemented with 100 mM NaCl and dialyzed for 1 h against the same buffer. This sample was then loaded onto a Q-Hiload (16 mm × 20 cm; Amersham) columm equilibrated in buffer A supplemented with 100 mM NaCl. The protein was recovered in the flow-through fraction (FT). This fraction was diluted 3-fold in buffer A and loaded onto a S-Sepharose high-performance columm (16 mm × 20 cm; Amersham). A 125 mL gradient from 33 to 500 mM NaCl in buffer A was then applied to the column. Fractions containing the protein were pooled, and the protein was concentrated by using a Centricon-30 concentrator (Amicon). The concentrated sample (3 mL) was finally loaded onto a Superdex75 columm (16 mm × 60 cm; Amersham) equilibrated in buffer A supplemented with 500 mM NaCl. The protein eluted in a single peak and was homogeneous as judged by SDS−PAGE analysis. The protein was concentrated to 250 $\mu$M by using a Centricon-30 concentrator. Protein concentration was deduced from $A_{280}$ measurements using an extinction coefficient of 0.437 $cm^2$/mg, computed from the amino acid sequence. Nearly 15 mg of protein per liter of culture was obtained.

A gene corresponding to domain IV of ss-aIF5B was constructed by PCR amplification of the cloned gene using primers introducing an *Nde*I site together with an ATG codon in place of the TTT codon specifying F462. The amplified *Nde*I−*Xho*I fragment was cloned between the corresponding sites of pET3alpa. A vector expressing domains I, II, and III of ss-aIF5B was constructed by site-directed mutagenesis of pET3a5Bss, resulting in the introduction of a stop (TGA) codon in place of the GAA codon at position 455. The two proteins were expressed and purified as described above, except that, in the case of domain IV, the protein was dialyzed for 1 h against buffer A before being loaded onto the S-Sepharose column. Protein concentrations were deduced from $A_{280}$ measurements using extinction coefficients of 0.378 and 0.463 for domain IV and for domains I, II, and III, respectively. These coefficients were calculated using the amino acid compositions of the polypeptides. Nearly 2 mg of domain IV or 10 mg of domains I, II, and III was obtained from 1 L cultures.

*pa-aIF5B.* The gene encoding pa-aIF5B is interrupted by an intein (394 codons) inserted after the 20th codon. The region corresponding to the C-terminal part of aIF5B (from residue 21) was PCR amplified from *P. abyssi* chromosomal DNA (*23*). The primers used added a *Sma*I site corresponding to G19 and K20 on one side, and a *Sal*I site on the other side. The *Sma*I−*Sal*I fragment was cloned between the *Sma*I and *Xho*I sites of pET3alpa to give pET3a5BpaC. The 5′ part of the gene was then reconstituted by cloning a synthetic oligonucleotide encoding codons 1−19 between the *Nde*I and *Sma*I sites of pET3a5BpaC. The resulting plasmid, pET3a5Bpa, exactly encodes mature pa-aIF5B. The protein was produced and purified as described above for ss-aIF5B, except that the streptomycin sulfate step was omitted and the starting NaCl concentration for the S-Sepharose step was 50 mM instead of 33 mM. The homogeneous protein was concentrated to 170 $\mu$M by using a Centricon-30 concentrator. Protein concentration was deduced from $A_{280}$ measurements using an extinction coefficient of 0.631 $cm^2$/mg, computed from the amino acid sequence. Nearly 8 mg of protein per liter of culture was obtained.

*y-ΔeIF5B.* y-ΔeIF5B corresponds to *Sa. cerevisiae* eIF5B deprived of its N-terminal extension of 400 amino acids. The gene encoding this protein was constructed by PCR amplification using DNA from yeast strain W303a, and oligonucleotide primers introducing an *Nde*I site together with an ATG codon in place of the codon for S395. The amplified *Nde*I−*Bam*HI fragment was cloned between the corresponding sites of pET15blpa. This expression vector was derived from pET15b (Novagen) by inserting the same polylinker as that used to design pET3alpa (see above).

The resulting plasmid, pET15b5By, encodes a $His_6$-tagged N-terminally truncated eIF5B. *E. coli* Rosetta pLacI-RARE cells containing pET15b5By were grown in 2× TY medium (1 L) containing ampicillin (50 $\mu$g/mL) and chloramphenicol (34 $\mu$g/mL). Expression was induced by adding IPTG to a final concentration of 1 mM when the $OD_{650}$ reached 1.6. The culture was then continued for 6 h at room temperature. Cells were disrupted by sonication in 40 mL of buffer B [10 mM MOPS (pH 6.7), 3 mM 2-mercaptoethanol, 0.1 mM PMSF, and 500 mM NaCl]. After centrifugation, the supernatant was loaded onto a column (5 mL) containing Talon affinity resin (Clontech). The column was washed with 50 mL of buffer B, and the protein was eluted with the same buffer supplemented with 25 mM imidazole. The eluate was

[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ss-aIF5B, aIF5B from *S. solfataricus*; pa-aIF5B, aIF5B from *P. abyssi*; y-eIF5B, eIF5B from *Sa. cerevisiae*; h-eIF5B, eIF5B from humans.

diluted 5-fold with buffer A and loaded onto a Q-Sepharose high-performance columm (16 mm × 20 cm; Amersham). An 80 mL gradient from 100 to 500 mM NaCl in buffer A was then applied to the column. Fractions containing the protein were pooled and submitted to concentration by using a Centricon-30 concentrator (Amicon). The concentrated sample (3 mL) was finally loaded onto a Superdex75 columm (16 mm × 60 cm; Amersham) equilibrated in buffer A supplemented with 500 mM NaCl. The protein eluted in a single peak and was homogeneous as judged by SDS−PAGE analysis. The protein was concentrated to 190 $\mu$M by using a Centricon-30 concentrator. Protein concentration was deduced from $A_{280}$ measurements using an extinction coefficient of 0.588 cm$^2$/mg, computed from the amino acid sequence. Nearly 22 mg of protein per liter of culture was obtained.

*tRNAs.* $tRNA_f^{Met}$, $tRNA_1^{Val}$, $tRNA^{Lys}$, and their variants were produced in *E. coli* from constructed genes, as described previously (*24*−*26*). The gene for $tRNA_i^{Met}$ from *S. solfataricus* was constructed by assembly of oligonucleotides and cloned between the *Eco*RI and *Pst*I sites of pBSTNAV2, as described previously (*27*). The cloned gene was then PCR amplified by adding *Nde*I and *Xho*I sites, and transferred into pET3alpa for expression in *E. coli* Rosetta pLacI-RARE cells. Overproduced tRNAs were purified by anion exchange chromatography (*27*). $tRNA_i^{Met}$ from *Sa. cerevisiae* was a kind gift from G. Keith (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France) (*28*). Amino acid acceptor capacities of all studied tRNAs were between 1000 and 1500 pmol/$A_{260}$ unit. Full aminoacylation of initiator tRNA with [$^{35}$S]methionine (10000 dpm/pmol, Perkin-Elmer) was achieved using homogeneous *E. coli* M547 MetRS (*29*). Valylation with [$^3$H]Val (5000 dpm/pmol, Amersham) was performed with homogeneous *E. coli* ValRS (*30*). Lysylation with [$^3$H]Lys (8000 dpm/pmol, Amersham) was performed with homogeneous *E. coli* LysRS (*26*). Obtained aminoacyl-tRNAs were precipitated with ethanol in the presence of 0.3 M NaAc (pH 5.5) and stored at −20 °C in water, in small aliquots. Before use, levels of aminoacylation of tRNAs were systematically verified after precipitation in 5% trichloroacetic acid (TCA).

*Protection Assay*

Protection of aminoacylated tRNAs against spontaneous hydrolysis by e/aIF5B was performed as follows (*31*). Reaction mixtures (120 $\mu$L) contained 20 mM Hepes-NaOH (pH 8.0), 100 mM KCl, 500 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.2 mg/mL BSA (bovine serum albumin, Roche), 5% glycerol, 1 mM GTP, and the aminoacylated tRNA under consideration (80 nM). Concentrations of aIF5B or of its variants were varied in the assay from 1 to 120 $\mu$M depending on the order of magnitude of the $K_d$ value to be measured. Unless otherwise stated, the mixtures were incubated at 51 °C. To determine rate constants of deacylation, 20 $\mu$L aliquots were withdrawn at various times (5 to 30 min) and precipitated in 5% TCA in the presence of 80 $\mu$g of yeast RNA as a carrier. Curves of deacylation as a function of time were obtained. In all cases, these curves could be fitted with a single-exponential function. Rate constants measured at variable protein concentrations were fitted to simple binding curves from which dissociation constants for studied protein−tRNA complexes were deduced using MC-Fit (*32*).

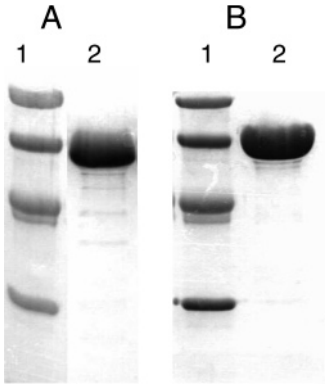


FIGURE 1: SDS−PAGE analysis of purified ss-aIF5B (A) or y-ΔeIF5B (B). Purified proteins (38 $\mu$g) were loaded onto 12% polyacrylamide gels (lane 2). Lane 1 contains molecular mass markers (Amersham; 94, 67, 43, and 30 kDa).

## RESULTS

*aIF5B Protects Initiator Met-tRNA against Deacylation.* In bacteria, binding of formyl-Met-$tRNA_f^{Met}$ to IF2 results in protection of the aminoacylated 3′-end of the tRNA ligand against spontaneous deacylation (*7*). By analogy with the bacterial system, we anticipated that any interaction of an initiator tRNA with aIF5B should be detected by a similar assay. The thermophilic archaeon *S. solfataricus* was chosen as a model system for performing such experiments. The gene encoding *S. solfataricus* aIF5B (ss-aIF5B) was cloned and expressed in *E. coli*. The ss-aIF5B factor was then purified to homogeneity (Figure 1). In addition, a gene corresponding to the initiator tRNA of *S. solfataricus* was overexpressed in *E. coli*. The product of this gene, $tRNA_i^{Met}$, was purified and aminoacylated with [$^{35}$S]methionine using *E. coli* methionyl-tRNA synthetase.

At 51 °C, deacylation of *S. solfataricus* Met-$tRNA_i^{Met}$ (80 nM) occurred spontaneously at a rate of 0.14 min$^{-1}$. In the presence of ss-aIF5B (50 $\mu$M) and GTP (1 mM), the deacylation rate was reduced to 0.01 min$^{-1}$. The deacylation rate varied as a function of the concentration of added ss-aIF5B. The saturation curve in Figure 2 indicates a dissociation constant of Met-$tRNA_i^{Met}$ from the ss-aIF5B−GTP−Met-$tRNA_i^{Met}$ complex equal to 2.7 ± 0.5 $\mu$M (Table 1, row 1). The value of this binding constant is comparable to that measured for the formyl-Met-$tRNA_f^{Met}$−IF2 complex (*7*, *10*). However, the tRNA binding affinity is much weaker than those observed with the tRNA-carrier factors e/aEF1A or e/aIF2 (*31*, *33*−*35*).

We also used the aIF5B factor of *P. abyssi* (pa-aIF5B) to follow protection of Met-$tRNA_i^{Met}$. In *P. abyssi*, the pa-aIF5B factor is derived from a precursor containing an intein. To overcome this difficulty, we designed a DNA fragment just corresponding to the pa-aIF5B coding sequence. This DNA construct was expressed in *E. coli*. Overexpressed pa-aIF5B was then purified to homogeneity. Using *P. abyssi* $tRNA_i^{Met}$ produced in *E. coli* (*31*), we observed efficient protection of Met-$tRNA_f^{Met}$ against deacylation in the presence of the pa-aIF5B factor. The dissociation constant of Met-$tRNA_i^{Met}$ for dissociation from its complex with pa-aIF5B and GTP was equal to 1.9 ± 0.4 $\mu$M (Table 1, row 2).

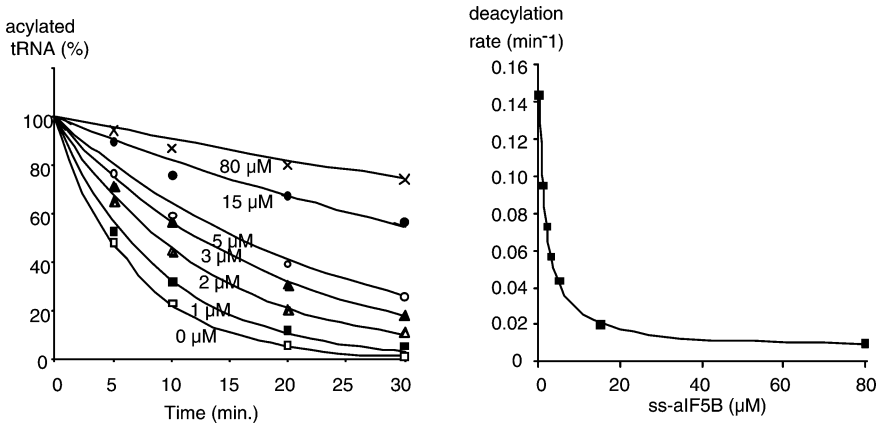


FIGURE 2: Determination of the dissociation constant ($K_d$) of *S. solfataricus* Met-tRNA$_i^{Met}$ from its complex with ss-aIF5B. The left panel shows kinetics of tRNA deacylation measured in the presence of a variable ss-aIF5B concentration. To derive deacylation rates, deacylation curves were fitted to single exponentials. Rates obtained at various protein concentrations are shown in the right panel. The set of data was fitted to a binding curve from which a dissociation constant of the aminoacyl-tRNA from its complex with ss-aIF5B was derived (Table 1; see Experimental Procedures).

Table 1: Dissociation Constants of e/aIF5B−Met-tRNA Complexes

| | protein | tRNA ligand | $K_d$[d] ($\mu$M) |
|---|---|---|---|
| 1 | ss-aIF5B | *S. solfataricus* Met-tRNA$_i^{Met}$ | 2.7 ± 0.5 |
| 2 | pa-aIF5B | *P. abyssi* Met-tRNA$_i^{Met}$ | 1.9 ± 0.4 |
| 3 | ss-aIF5B | Met-tRNA$_f^{Met}$ | 4.2 ± 0.7 |
| 4 | pa-aIF5B | Met-tRNA$_f^{Met}$ | 2.7 ± 0.7 |
| 5 | ss-aIF5B[a] | Met-tRNA$_f^{Met}$ | 3.5 ± 0.7 |
| 6 | ss-aIF5B[b] | Met-tRNA$_f^{Met}$ | 3.9 ± 0.7 |
| 7 | ss-aIF5B | Met-tRNA$_1^{Val}$CAU | 2.7 ± 0.5 |
| 8 | ss-aIF5B | Met-tRNA$_m^{Met}$ | 3.3 ± 0.5 |
| 9 | ss-aIF5B domain IV | Met-tRNA$_f^{Met}$ | 1.6 ± 0.3 |
| 10 | ss-aIF5B domains I, II, and III | Met-tRNA$_f^{Met}$ | >80 |
| 11 | y-ΔeIF5B[c] | Met-tRNA$_f^{Met}$ | 40 ± 5 |
| 12 | y-ΔeIF5B[c] | *Sa. cerevisiae* Met-tRNA$_i^{Met}$ | 41 ± 10 |
| 13 | y-ΔeIF5B[c] | Lys-tRNA$^{Lys}$ | 110 ± 50 |
| 14 | y-ΔeIF5B[c] | Val-tRNA$_1^{Val}$ | 140 ± 70 |
| 15 | y-ΔeIF5B[c] | Met-tRNA$_1^{Val}$CAU | 46 ± 10 |

[a] Measured in the presence of 1 mM GDP. [b] Measured in the presence of 1 mM GDPNP. [c] Measured at 30 °C. [d] Each dissociation constant was derived from the measurement of tRNA deacylation rates in the presence of various protein concentrations, as described in Experimental Procedures. Unless otherwise stated, all experiments were carried out in the presence of 1 mM GTP.

Initiator tRNAs from *S. solfataricus* or *P. abyssi* closely resemble that from *E. coli*. The sequences of the acceptor stems are identical, the only exception being a weak $A_1 \cdot U_{72}$ base pair in the archaeal tRNAs instead of unpaired bases 1 and 72 found in bacterial tRNAs. Despite this difference, *E. coli* Met-tRNA$_f^{Met}$ bound ss-aIF5B with an affinity ($K_d$ = 4.2 ± 0.7 $\mu$M) similar to that observed with *S. solfataricus* initiator tRNA produced in *E. coli* (Table 1, rows 1 and 3). *E. coli* Met-tRNA$_f^{Met}$ also bound pa-aIF5B with an affinity ($K_d$ = 2.7 ± 0.5 $\mu$M) similar to that observed with *P. abyssi* initiator tRNA (Table 1, rows 2 and 4). We therefore concluded that *E. coli* Met-tRNA$_f^{Met}$ was a valuable model ligand for further studying formation of a complex between ss-aIF5B and tRNA.

Binding of tRNA by bacterial IF2 does not change upon substitution of GTP with GDP (*7*). Therefore, we followed protection of Met-tRNA$_f^{Met}$ by ss-aIF5B in the presence of GDP or of the nonhydrolyzable GTP analogue, GDPNP. In both cases, tRNA binding constants were identical to that measured in the presence of GTP (Table 1, rows 3, 5, and 6). We therefore concluded that, as in the case of bacterial IF2, binding of methionylated initiator tRNA by aIF5B does not depend on the nucleotidic state of the factor.

*aIF5B Recognizes the Methionyl Moiety of Its tRNA Ligand.* To evaluate the specificity of aminoacyl-tRNA protection by ss-aIF5B, several potential ligands were used in the protection assay. First, two *E. coli* non-methionine aminoacyl-tRNAs, Lys-tRNA$^{Lys}$ and Val-tRNA$_1^{Val}$, were assayed for their capacity to bind ss-aIF5B. Slight protection of Lys-tRNA$^{Lys}$ could be obtained at the highest concentration of ss-aIF5B that was used (80 $\mu$M, Figure 3). From this experiment, we estimated the dissociation constant of the ss-aIF5B−Lys-tRNA$^{Lys}$ complex to be on the order of 50 $\mu$M. The rate of spontaneous deacylation of Val-tRNA$_1^{Val}$ at 51 °C (0.03 min$^{-1}$) was quite slow. However, in the presence of 100 mM Bicine-NaOH (pH 9.0), the deacylation rate of Val-tRNA$_1^{Val}$ could be increased to 0.17 min$^{-1}$. Therefore, we chose to study protection of this tRNA by ss-aIF5B at pH 9.0, to obtain a better precision in the measurements of the deacylation rates. Under these conditions, slight but significant protection of Val-tRNA$_1^{Val}$ was observed in the presence of 80 $\mu$M ss-aIF5B (Figure 3). The dissociation constant of the ss-aIF5B−Val-tRNA$^{Val}$ complex can be roughly estimated to be on the order of 50 $\mu$M. Under the same buffer conditions, Met-tRNA$_f^{Met}$ bound ss-aIF5B with a $K_d$ of 4.2 ± 0.7 $\mu$M, identical to that measured at pH 8.0.

To identify the origin of the weaker binding of the two tRNAs described above to ss-aIF5B, two genetically engineered tRNAs were used. The first one, tRNA$_f^{Met}$UAC, is derived from tRNA$_f^{Met}$. It carries a Val (UAC) anticodon that allows it to be efficiently aminoacylated by valyl-tRNA synthetase (*36*). The second one, tRNA$_1^{Val}$CAU, is derived from tRNA$_1^{Val}$ by the introduction of a Met (CAU) anticodon. It can therefore be readily aminoacylated with methionine. Weak protection of Val-tRNA$_f^{Met}$UAC by ss-aIF5B was observed ($K_d$ can be roughly estimated to be on the order of 50 $\mu$M, Figure 3). We conclude, therefore, that, with a valine instead of a methionine, the binding affinity of aminoacylated initiator tRNA is reduced by 1 order of magnitude. On the other hand, although being different from initiator tRNA in its nucleotide sequence, Met-tRNA$_1^{Val}$CAU was efficiently protected by ss-aIF5B against deacylation ($K_d$ = 2.7 ± 0.5 $\mu$M; Figure 3 and Table 1, row 7), thus showing the

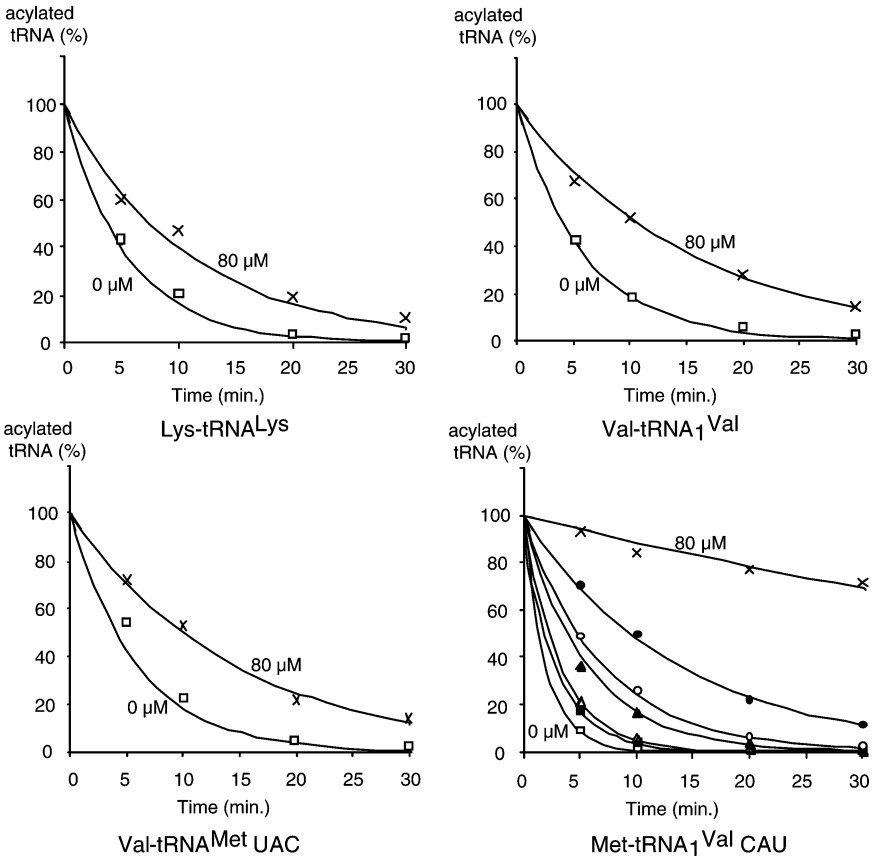


FIGURE 3: Specificity of aminoacyl-tRNA binding by ss-aIF5B. The first three panels show the kinetics of deacylation of the indicated aminoacyl-tRNA (Lys-tRNA$^{Lys}$, Val-tRNA$_1^{Val}$, or Val-tRNA$^{Met}$UAC) measured in the presence of 80 $\mu$M ss-aIF5B ($\times$) or in the absence of the factor ($\square$). In the case of Met-tRNA$^{Val}$C34A35U36, additional deacylation curves were followed in the presence of several other ss-aIF5B concentrations [($\blacksquare$) 1, ($\triangle$) 2, ($\blacktriangle$) 5, ($\bigcirc$) 8, and ($\bullet$) 15 $\mu$M]. These curves are shown in the fourth panel. Measurements with valylated tRNAs were taken in the presence of 100 mM Bicine-NaOH (pH 9.0) buffer.

importance of the methionyl moiety in the recognition by the factor. In agreement with this conclusion, *E. coli* elongator tRNA$_m^{Met}$ carrying an esterified methionine bound ss-aIF5B with a $K_d$ of 3.3 $\pm$ 0.5 $\mu$M (Table 1, row 8). Together, the results given above show that ss-aIF5B has the capacity to distinguish between various potential aminoacyl-tRNA ligands, and that its specificity is essentially directed toward the methionyl moiety.

*Domain IV of aIF5B Is Sufficient for Met-tRNA Binding.* The crystal structure of aIF5B from *Methanococcus jannaschii* indicates four domains (*15*). Domain I corresponds to the G domain. It is followed by a $\beta$-barrel (domain II) and by an $\alpha/\beta/\alpha$-sandwich (domain III). The latter domain is connected to another $\beta$-barrel (domain IV), via a long $\alpha$-helix. In the case of bacterial IF2, the isolated C-terminal $\beta$-barrel was shown to bind formyl-Met-tRNA$_f^{Met}$ with the same affinity and specificity as full-length IF2. This prompted us to genetically produce ss-aIF5B fragments and to follow their ability to bind Met-tRNA$_f^{Met}$. An N-terminal fragment corresponding to domains I, II, and III (residues 1−454) and a C-terminal one corresponding to domain IV (from residue 463) were prepared. As shown in Table 1 (row 10), deletion of the C-terminal domain abolished the capacity of ss-aIF5B to protect Met-tRNA$_f^{Met}$ against deacylation. In contrast, isolated domain IV was sufficient to restore full protection (Figure 4), with a binding affinity of Met-tRNA$_f^{Met}$ comparable to that measured with full-length ss-aIF5B (Table 1, row 9). Domain IV also weakly protected Val-tRNA$_1^{Val}$ or Val-tRNA$_f^{Met}$CUA against deacylation (Figure 4). We concluded therefore that, as in the case of IF2, the C-terminal domain of aIF5B is fully responsible for aminoacyl-tRNA binding.

*Protection of Initiator Met-tRNA by Yeast eIF5B.* Eukaryotic eIF5B differs from its archaeal aIF5B counterpart by the presence of a long N-terminal extension (400 residues in *Sa. cerevisiae* y-eIF5B, 600 residues in human eIF5Bh). However, archaeal aIF5B succeeds in partially compensating for the absence of its yeast eIF5B homologue both in vivo and in vitro (*37*). Therefore, it is very likely that the archaeal and eukaryotic factors share the same mode of action. Actually, the dispensability of the N-terminal extension of eukaryotic eIF5B is indicated by the observation that N-terminally truncated forms of y-eIF5B and h-eIF5B rescue the growth defect of a yeast strain knocked out for its eIF5B gene (*37*). Moreover, His$_6$-tagged N-terminally truncated eIF5By is fully functional in an in vitro methionylpuromycin synthesis assay using yeast 80S ribosomes and initiation factors (*17*).

In this context, we wondered whether Met-tRNA$_f^{Met}$ could associate with yeast y-eIF5B. Despite several attempts, we could not produce full-length y-eIF5B in *E. coli* from its cloned gene. In contrast, we succeeded in overproducing a His$_6$-tagged N-terminally truncated form of y-eIF5B (y-$\Delta$eIF5B, Figure 1; see Experimental Procedures). At high concentrations, the y-$\Delta$eIF5B factor significantly protected *E. coli* Met-tRNA$_f^{Met}$ against deacylation (Figure 5). At the highest assayed concentration (122 $\mu$M), the deacylation rate reached a value of 0.01 min$^{-1}$. For comparison, under the

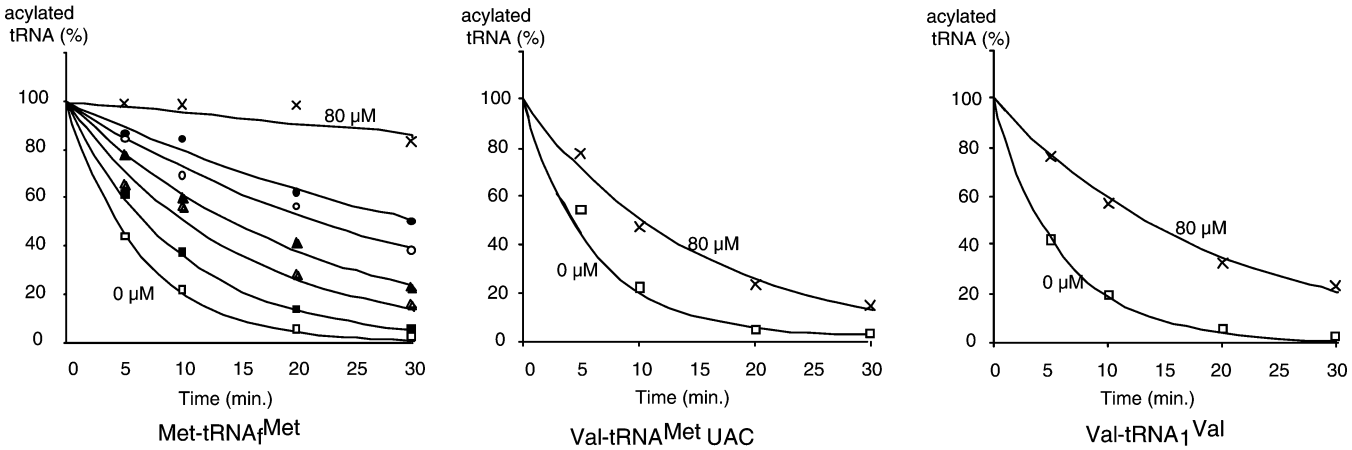


FIGURE 4: Aminoacyl-tRNA binding by the isolated domain IV of ss-aIF5B. Each panel shows the kinetics of deacylation of the indicated aminoacyl-tRNA measured in the presence of 80 $\mu$M domain IV of ss-aIF5B (×) or in the absence of the domain (□). In the case of Met-tRNA$_f^{Met}$, deacylation curves in the presence of several other domain IV concentrations are shown [(■) 1, (△) 2, (▲) 5, (○) 8, and (●) 15 $\mu$M]. Measurements with valylated tRNAs were taken in the presence of 100 mM Bicine-NaOH (pH 9.0) buffer.

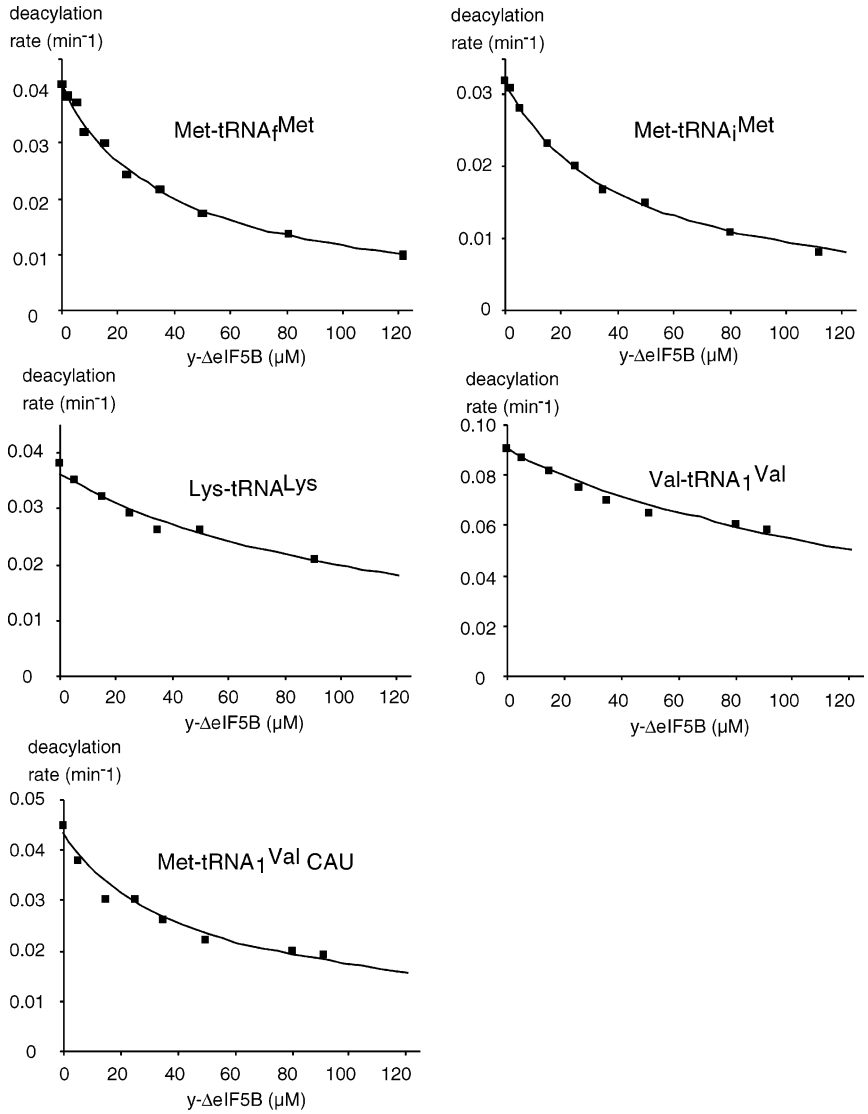


FIGURE 5: Aminoacyl-tRNA binding by y-ΔeIF5B. Panels show plots of the deacylation rates obtained with five aminoacyl-tRNAs (*E. coli* Met-tRNA$_f^{Met}$, *Sa. cerevisiae* Met-tRNA$_i^{Met}$, *E. coli* Lys-tRNA$^{Lys}$, *E. coli* Val-tRNA$_1^{Val}$, and *E. coli* Met-tRNA$_1^{Val}$CAU) as a function of y-ΔeIF5B concentration. The five sets of data were fitted to binding curves from which dissociation constants of the various aminoacyl-tRNAs from their complexes with y-ΔeIF5B were derived (Table 1; see Experimental Procedures). Experiments were carried out at 30 °C.

same experimental conditions (30 °C), the rate of spontaneous deacylation of Met-tRNA$_f^{Met}$ was 0.04 min$^{-1}$. As a further control, we verified that the addition of 80 $\mu$M bovine serum albumin instead of y-ΔeIF5B had only a slight effect

on the deacylation rate (0.036 $min^{-1}$). By using the variation of the deacylation rate as a function of the concentration of the factor, we measured a dissociation constant of 40 ± 5 $\mu$M (Figure 5 and Table 1, row 11). A similar value was measured at 25 °C (39 ± 4 $\mu$M).

Yeast initiator tRNA carries several post-transcriptional modifications, including a specific 2′-*O*-ribosyl phosphorylation on base 64 (*38*, *39*). To rule out possible artifacts related to the use of *E. coli* initiator tRNA in our experiments, the protection experiment was repeated using authentic $tRNA_i^{Met}$ extracted from *Sa. cerevisiae* cells. The measured dissociation constant, 41 ± 10 $\mu$M, was identical to that recorded with Met-$tRNA_f^{Met}$ (Table 1, row 12). Finally, the specificity of tRNA binding by y-ΔeIF5B was assessed by using *E. coli* elongator Lys-$tRNA^{Lys}$ and Val-$tRNA_1^{Val}$. Protection of these aminoacyl-tRNAs against deacylation was observed (Figure 5). $K_d$ values were in the range of 100−150 $\mu$M (Table 1, rows 13 and 14). With Met-$tRNA_1^{Val}$CAU, the dissociation constant, 46 ± 10 $\mu$M, was similar to that measured with initiator Met-$tRNA_i^{Met}$ (Figure 5 and Table 1, row 15). Therefore, aminoacyl-tRNA binding by y-ΔeIF5B appears not to depend on the sequence of the tRNA that is used. In addition, although an esterified methionine confers some specificity in the binding to the factor, the advantage given by this amino acid is less pronounced than in the case of archaeal aIF5B.

## DISCUSSION

In bacteria, selection of the initiator tRNA from the pool of cellular tRNAs is mainly achieved by IF2, through recognition of the N-blocked methionine esterified to this tRNA (*6*, *8*, *9*), and also through recognition of the top of the acceptor stem of this tRNA (*9*). The stability of the complex between IF2 and fMet-$tRNA_f^{Met}$ does not depend on the nucleotidic state of the factor, bound to GTP or to GDP (*7*). Once it has reached the P site on the 30S subunit, the initiator tRNA is better stabilized by the GTP form of 30S-bound IF2 than by the GDP form (*16*). This behavior indicates that IF2 plays specific roles in the ribosome, which is consistent with its documented role in joining of subunits.

In archaea as well as in eukarya, selection of the initiator tRNA is achieved by the heterotrimeric factor e/aIF2, which binds Met-$tRNA_i^{Met}$ with a dissociation constant in the nanomolar range. However, genetic data indicate that e/aIF5B also participates in initiator tRNA recruitment (*21*). Moreover, as for IF2, eIF5B promotes joining of the ribosomal subunits during the final steps of translation initiation (*17*).

This study now presents evidence that, in a manner analogous to that observed with IF2, e/aIF5B recognizes the methionylated 3′-end of initiator tRNA. As in IF2, archaeal aIF5B binds Met-$tRNA_i^{Met}$ with a $K_d$ in the micromolar range. *Sa. cerevisiae* eIF5B displays less affinity for its initiator tRNA ($K_d$ = 40 $\mu$M). The analogy between e/aIF5B and IF2 is strengthened by the fact that, in both cases, the C-terminal $\beta$-barrel domain binds the initiator tRNA with an affinity equal to that measured with the full-length protein (refs *10*−*12* and this study). Therefore, our results reinforce the idea that IF2 and e/aIF5B have kept some constant function(s) during evolution (*5*, *21*). One possible common function is verification of the presence of a correctly positioned initiator tRNA in the ribosomal P site before the two ribosomal subunits join together (*14*, *16*, *19*, *20*). IF2 and e/aIF5B would therefore be engaged in a final checkpoint, before translation can begin (*14*, *20*).

Contrasting with bacterial IF2, which displays strong specificity toward the N-blocked methionine esterified to the initiator tRNA, archaeal aIF5B shows a reduced specificity toward the methionyl moiety of the initiator tRNA. Moreover, it appears to be indifferent to the nucleotide sequence of this tRNA. In the case of *Sa. cerevisiae* eIF5B, the specificity toward methionine is very weak. Such a relatively poor specificity of eIF5B and, to a lesser extent, of aIF5B is consistent with the idea that, in both eukarya and archaea, primary selection of the initiator tRNA is governed with high affinity by e/aIF2. Actually, in eukarya, numerous initiation factors guarantee the selection and channeling of the initiator tRNA. In this context, the only plausible function of eIF5B should be at the level of the ribosome. Possibly, eIF5B is recruited when an aminoacylated tRNA occupies the P site (*19*). This recruitment would occur whatever the nature of this aminoacyl-tRNA. This might explain why, in *Sa. cerevisiae*, disruption of the gene for eIF5B only causes a slow growth phenotype (*21*), while eIF2 is strictly essential for cell viability (*40*). In archaea, because of a smaller number of factors participating in initiation of translation, primary selection of the initiator tRNA may be less stringent. One can therefore imagine that a selective advantage has been confered to an aIF5B factor exerting more specificity toward methionine at the P site than eIF5B does.

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