Translation initiation complex formation in the crenarchaeon *Sulfolobus solfataricus*

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ABSTRACT

The function of initiation factors in and the sequence of events during translation initiation have been intensively studied in Bacteria and Eukaryotes, whereas in Archaea knowledge on these functions/processes is limited. By employing chemical probing, we show that translation initiation factor aIF1 of the model crenarchaeon *Sulfolobus solfataricus* binds to the same area on the ribosome as the bacterial and eukaryal orthologs. Fluorescence energy transfer assays (FRET) showed that aIF1, like its eukaryotic and bacterial orthologs, has a fidelity function in translation initiation complex formation, and that both aIF1 and aIF1A exert a synergistic effect in stimulating ribosomal association of the Met-tRNA^{Met} binding factor a/eIF2. However, as in Eukaryotes their effect on a/eIF2 binding appears to be indirect. Moreover, FRET was used to analyze for the first time the sequence of events toward translation initiation complex formation in an archaeal model system. These studies suggested that a/eIF2-GTP binds first to the ribosome and then recruits Met-tRNA^{Met}, which appears to comply with the operational mode of bacterial IF2, and deviates from the shuttle function of the eukaryotic counterpart eIF2. Thus, despite the resemblance of eIF2 and a/eIF2, recruitment of initiator tRNA to the ribosome is mechanistically different in Pro- and Eukaryotes.

Keywords: Archaea; *Sulfolobus solfataricus*; translation initiation; aIF1; a/eIF2

INTRODUCTION

Protein synthesis in Prokaryotes starts with the interaction of initiator fMet-tRNA^{Met}, mRNA, and the small (30S) ribosomal subunit. The two ligands can bind randomly to the 30S ribosomal subunit without initially interacting with each other, yielding a 30S pre-initiation complex (Gualerzi and Pon 1990). A rate-limiting conformational change, which is kinetically controlled by all three initiation factors IF1, IF2, and IF3, converts the 30S pre-initiation complex into a 30S initiation complex (Gualerzi and Pon 1990; Gualerzi et al. 2001). During this transition the initiation codon of the mRNA moves from an initial “standby” site to the P-decoding site, where it base-pairs with the anticodon of fMet-tRNA^{Met} (La Teana et al. 1995; Yusupova et al. 2006; Kaminishi et al. 2007). The trans-

lation initiation factors IF1, IF2, and IF3 have different roles in facilitating 30S initiation complex formation (Laursen et al. 2005). IF3 affects both the on and off rates of fMet-tRNA^{Met} binding and introduces a translational bias that impedes translational starts from noncanonical initiation complexes (Gualerzi and Pon 1990; Gualerzi et al. 2001). Recent studies have shown that IF3 release from the ribosome occurs during formation of the 70S initiation complex, i.e., during joining of the 50S subunit with the 30S initiation complex (Milon et al. 2008). IF2 has a complementary role to that of IF3, selectively promoting ribosomal binding of fMet-tRNA^{Met} by increasing its on-rate (Gualerzi et al. 2001). It has been a matter of debate whether fMet-tRNA^{Met} binds to 30S subunits as part of a binary complex with IF2 (Mayer et al. 2003), or whether fMet-tRNA^{Met} is recruited by 30S-bound IF2 (Boelens and Gualerzi 2002). Recent fluorescence energy transfer assays (FRET) argue for the latter mode of action (M Carotti and CO Gualerzi, unpubl.). IF1 was shown to bind to the A-site of the 30S subunit, thereby preventing premature A-site binding of elongator tRNA (Moazed et al. 1995).

Translation initiation in Eukaryotes starts with the assembly of the eIF2-GTP•Met-tRNA^{Met} ternary complex (Richter and Lipmann 1970). After its formation, the

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ternary complex binds to the 40S ribosomal subunit. This binding is facilitated by the initiation factors eIF1, eIF1A, and eIF3, resulting in the 43S complex. On the other hand the eIF4F complex assembles on the 5'-cap of the mRNA. eIF4F in conjunction with eIF3 and the poly(A) binding protein bound to the 3'-tail of the mRNA then by virtue of protein-protein interactions loads the mRNA onto the 43S complex (for recent reviews, see Kapp and Lorsch 2004; Acker and Lorsch 2008; Sonenberg and Hinnebusch 2009). The 43S complex then scans along the mRNA in 5'→3' direction in search for the start codon. Cryoelectron microscopy studies suggested that both eIF1 and eIF1A maintain the initiating ribosome in an "open" scanning competent conformation until the start codon is located, and start-codon/anti-codon base-pairing has been established (Passmore et al. 2007). As a result of a conformational change in the complex eIF1 is released (Maag et al. 2005; Cheung et al. 2007). The release of eIF1 could free the C terminus of eIF1A for interactions with eIF5, which stabilizes the closed state of the complex (Maag et al. 2006). The final step in translation initiation includes joining of the 60S ribosomal subunit to the 43S pre-initiation complex. The factor eIF5B facilitates subunit joining, whereby GTP hydrolysis is not required for subunit joining per se, but for the release of eIF5B after subunit joining has occurred (Pestova et al. 2000; Shin et al. 2002).

Archaea possess more translation initiation factors than Bacteria, but lack some of the eukaryotic ones, including those present in the cap complex, eIF4F, eIF3, and the GDP-GTP exchange factor for eIF2. The best-studied translation initiation factor is a/eIF2, which is pivotal for Met-tRNAi^{Met} binding to the ribosome (Yatime et al. 2004; Pedulla et al. 2005). Several 3D structures are available for a/eIF2, providing information on the general shape of the trimeric factor and Met-tRNAi^{Met} binding (Yatime et al. 2006; Nikonov et al. 2007; Yatime et al. 2007). Recent studies showed that aIF1 promotes formation of the 30S•a/eIF2-GTP•Met-tRNAi^{Met} complex (Hasenöhrl et al. 2006). In addition to its Met-tRNAi^{Met} binding function, a/eIF2, in particular the a/eIF2 γ -subunit, was shown to bind with high affinity to the triphosphorylated 5' end (5'-P₃-end) of mRNA, leading to protection from 5'→3' mRNA decay. In addition, this study indicated that the ratio between the a/eIF2 γ levels and 30S subunits increase under starvation conditions, which was paralleled by an increased in vivo stability of the 5' segment of *Sulfolobus solfataricus* 2508 mRNA (Hasenöhrl et al. 2008). Another archaeal factor with an assigned function is aIF5B. The protein functions as a ribosome-dependent GTPase, which is important for translation of both leaderless and canonical mRNAs (Maone et al. 2007). In *S. solfataricus* and other Crenarchaeota, mRNAs are often bicistronic. The first cistron frequently encodes a leaderless mRNA, at which the 5'-terminal codon either serves as the start codon or the start codon is preceded by only a few nucleotides. In con-

trast, internal cistrons are generally preceded by a Shine-Dalgarno (SD) sequence (Tolstrup et al. 2000).

In contrast to bacterial (McCutcheon et al. 1999; Carter et al. 2001; Dallas and Noller 2001; Marzi et al. 2003) and eukaryal factors (Lomakin et al. 2003; Fraser et al. 2007; Unbehaun et al. 2007), the localization of archaeal translation initiation factors on the ribosome is unknown. Here, we present the first probing data for *S. solfataricus* aIF1 on the 30S subunit, provide evidence for a fidelity function of aIF1, and show that this factor in conjunction with aIF1A stimulates binding of a/eIF2 to the 30S ribosomal subunit. Moreover, we address the question of the sequence of events during assembly of the translation initiation complex in *S. solfataricus* by scrutinizing whether a ternary a/eIF2-GTP•Met-tRNAi^{Met} complex—like in Eukaryotes—shuttles Met-tRNAi^{Met} to the 30S ribosome, or whether a/eIF2 binds first to 30S and then recruits Met-tRNAi^{Met}.

RESULTS

Localization of aIF1 on the *S. solfataricus* 30S ribosomal subunit

Hydroxyl radical probing was used to study the interaction between *S. solfataricus* translation initiation factor aIF1 and the 30S ribosomal subunit. *S. solfataricus* 30S subunits and aIF1 contained in two separate syringes were mixed in a quench flow apparatus. After an incubation period of 2 sec, Fe(II)-EDTA was added, and the cleavage reaction was allowed to proceed for 20–30 msec before being quenched. The cleavage pattern of the entire 16S rRNA was analyzed in the absence or presence of aIF1 by primer extension using primers covering the whole 16S rRNA. As shown in Figure 1, aIF1 protected nucleotides 658/659 (Fig. 1A,C) and 749/750 (Fig. 1B,D) in helices 23 and 24, respectively, both of which are located at the subunit interface of the 30S ribosomal subunit (Spahn et al. 2001). Chemical probing experiments in the presence of both aIF1 and aIF1A revealed footprints in helices 23 and 24 indistinguishable from those obtained in the presence of only aIF1 (data not shown). The presence of both, aIF1 and aIF1A, protected additionally nucleotides 1011–1012 in helix 34 (Supplemental Fig. S1). However, chemical probing with aIF1A alone, unlike aIF1, did not affect the hydroxyl radical cleavage pattern of 16S rRNA (data not shown). We therefore consider the protection observed in helix 34 by aIF1/aIF1A to result from conformational changes upon binding of aIF1A or both factors rather than from a direct shielding of this region by aIF1A.

According to these data, aIF1 binds to the 30S ribosomal subunit at an analogous position as the eukaryotic ortholog eIF1 (Lomakin et al. 2003) and the presumed functional homolog IF3 (Dallas and Noller 2001). The similar topographical localization indicated that these factors could have similar functions, i.e., a role in (1) avoidance of

FIGURE 1. Hydroxyl radical probing of 16S rRNA in 30S/aIF1 complexes. (A,B) Primer extension analysis of helices 23 (A) and 24 (B) in 16S rRNA in 30S/aIF1 complexes upon hydroxyl radical probing. The individual lanes are indicated: 16S rRNA sequencing (T, A, C, and G), 30S modified in the absence of the factor (30S) or in the presence of the stoichiometric excess of aIF1 (2×, 5×, and 10×, as indicated). (C,D) Elements of helices 23 and 24 of 16S rRNA with protected sites shown by bars.

premature subunit joining during translation initiation, (2) acceleration of on and off kinetics of translation initiation complex formation, (3) increasing the ribosome affinity for other translational components, and (4) ensuring a fidelity function in translation initiation start-codon selection.

aIF1 discriminates against a noncanonical start codon

To assess whether aIF1 acts as a fidelity factor in translation initiation, mRNA binding to the 30S subunit was analyzed by fast kinetics employing a fluorescence energy transfer (FRET) assay. The FRET signal resulting from binding of fluorescein-labeled mRNA to a pre-assembled complex consisting of 30S, fluorescently labeled a/eIF2₅₆₅, GTP, Met-tRNAi^{Met}, and aIF1A was measured in the presence and absence of aIF1, and as a function of the nature of the initiation triplet present on the mRNA. Two types of mRNA-oligonucleotides were used, one containing the

canonical AUG triplet (AUG-mRNA_F) and the other the noncanonical AUU triplet (AUU-mRNA_F). As shown in Figure 2A, in the presence of aIF1 the binding of the AUG-mRNA_F to the ribosome was increased, while that of the AUU-mRNA_F was strongly diminished. This discrimination by aIF1 was observed only in the presence of Met-tRNAi^{Met}, while in its absence aIF1 had no effect on binding of either AUG-mRNA_F or the AUU-mRNA_F (Fig. 2B). This finding indicated that the fidelity function of aIF1 depends on the start-codon/anti-codon interaction.

aIF1 and aIF1A stimulate binding of a/eIF2 to the ribosome

It has been recently shown that aIF1 stimulates translation in *S. solfataricus* (Hasenöhrl et al. 2006). To test whether this results from a stimulation of ribosomal binding of a/eIF2 by aIF1 and/or aIF1A, we took advantage of the fluorescence spectral change which occurs upon binding of fluorescently labeled a/eIF2₅₆₅ to *S. solfataricus* 30S ribosomal subunits or to 30S particles pre-incubated with aIF1 and/or aIF1A. Initial experiments revealed that the kinetics of binding to 30S subunits were identical for a/eIF2₅₆₅, a/eIF2₅₆₅-GTP, and a/eIF2₅₆₅-GTP•Met-tRNAi^{Met} (data not shown). As shown in Figure 3A, binding of a/eIF2₅₆₅ to 30S subunits pre-incu-

bated with aIF1 (blue trace), aIF1A (green trace), or both aIF1 and aIF1A (red trace) was faster when compared with binding of a/eIF2₅₆₅ to the 30S ribosomal subunit in the absence of aIF1/aIF1A (orange trace). A two-exponential fit was used to analyze the data. The k_{on} values for binding of a/eIF2 to 30S ribosomes and to the 30S•aIF1•aIF1A complex were calculated with $12 \pm 0.5 \mu\text{M}^{-1}\text{sec}^{-1}$ and $67 \pm 3 \mu\text{M}^{-1}\text{sec}^{-1}$, respectively (Fig. 3B). The y -axis intercept yielded dissociation rate constants (k_{off}) of $1.07 \pm 0.4 \text{ sec}^{-1}$ for the 30S•aIF1•aIF1A complex and of $0.76 \pm 0.01 \text{ sec}^{-1}$ for 30S ribosomes. The equilibrium binding constant for binding of a/eIF2 resulted in a $K_d = 0.015 \pm 0.007 \mu\text{M}$ for the 30S•aIF1•aIF1A complex and $0.06 \pm 0.005 \mu\text{M}$ for the 30S subunit alone (Fig. 3B). The concentration-dependent analysis of k_{app1} and k_{app2} indicated that the fast reaction (k_{app2}) reflects binding and the slow reaction (k_{app1}) the adjustment of binding.

Far-Western blotting and co-immunoprecipitation did not reveal any direct interaction between aIF1 and a/eIF2 in

FIGURE 2. Discrimination by aIF1 against a non-AUG start codon. (A) AUG-mRNA_F binding to 30S•aIF1•a/eIF2₅₆₅-GTP•Met-tRNA^{Met}_i in the absence (green trace) and presence (red trace) of aIF1. AUU-mRNA_F binding to 30S•aIF1•a/eIF2₅₆₅-GTP•Met-tRNA^{Met}_i in the absence (blue trace) and presence (orange trace) of aIF1. Control measurements were performed with unlabeled AUG-mRNA and labeled a/eIF2₅₆₅ (black trace, acceptor only) or with labeled AUG-mRNA_F and unlabeled a/eIF2 (gray trace, donor only). (B) AUG-mRNA_F binding to 30S•aIF1•a/eIF2₅₆₅-GTP in the absence of Met-tRNA^{Met}_i and in the presence (red trace) or absence (green trace) of aIF1. AUU-mRNA_F binding to 30S•aIF1•a/eIF2₅₆₅-GTP in the absence (blue trace) and presence (orange trace) of aIF1. The charts in A and B show the k_{av} values determined as described in Materials and Methods.

solution (Supplemental Fig. S2). In addition, single cysteine mutants of aIF1 (the cysteine at position 6 was substituted by alanine, leaving only one cysteine at position 14) and aIF1A (the cysteines at position 25 and 40 were substituted by alanines, leaving only the cysteine at position 78) were labeled with Atto488. In the absence of *S. solfataricus* 30S ribosomal subunits no FRET signals were obtained between aIF1₄₈₈ or aIF1A₄₈₈ and a/eIF2₅₆₅, respectively (data not shown). Taken together, these data did not favor a direct stimulation of a/eIF2 binding to the 30S ribosomal subunit by aIF1 and/or aIF1A.

Order of events toward formation of the archaeal translation-initiation complex

It has been recently observed that the free a/eIF2 trimer has a higher affinity for the 5' triphosphate end (5'-P₃) of mRNAs than for Met-tRNA^{Met}_i (Hasenöhrl et al. 2008). Since binding of Met-tRNA^{Met}_i to *S. solfataricus* ribosome strictly requires the presence of a/eIF2 (Yatime et al. 2004;

Pedulla et al. 2005), this finding was at first glance difficult to reconcile with a Met-tRNA^{Met}_i shuttling function of a/eIF2, as known for the eukaryotic factor eIF2 (Kapp and Lorsch 2004). In fact, a preferential binding of a/eIF2 to mRNA rather than to Met-tRNA^{Met}_i would be expected to interfere with translation initiation. However, competition experiments revealed that, while increasing concentrations of 5'-P₃-RNA released Met-tRNA^{Met}_i from an a/eIF2-GTP•Met-tRNA^{Met}_i complex, they hardly competed away the Met-tRNA^{Met}_i bound to a 30S•a/eIF2-GTP complex. Moreover, when 5'-P₃-mRNA was added to a 30S•aIF1•a/eIF2-GTP•Met-tRNA^{Met}_i complex, Met-tRNA^{Met}_i was not released (Hasenöhrl et al. 2008). Thus, reasoning that the apparent competition between the 5'-P₃-RNA and Met-tRNA^{Met}_i for a/eIF2 would be alleviated if (1) a/eIF2-GTP would associate faster with 30S subunits than with 5'-P₃-RNA, and (2) 30S-bound a/eIF2-GTP would have a higher affinity for Met-tRNA^{Met}_i than for 5'-P₃-RNA, we determined the k_{on} of a/eIF2₅₆₅-GTP for Met-tRNA^{Met}_i and for the 5'-P₃-end of mRNA. As a/eIF2 binding was

FIGURE 3. aIF1 and aIF1A stimulate a/eIF2 binding to *S. solfataricus* 30S ribosomal subunits. (A) a/eIF2₅₆₅ binding to 30S ribosomal subunits (orange trace) and in the presence of 30S-bound aIF1A (green trace), aIF1 (blue trace), and aIF1/aIF1A (red trace), respectively. The chart shows the A_1 , k_{app1} , A_2 , k_{app2} , and k_{av} values determined by a two-exponential fitting as described in Materials and Methods. (B) The k_{on} values for a/eIF2₅₆₅ binding to 30S•aIF1•aIF1A complexes (▼) and to 30S subunits (■) were determined by titration of the respective k_{app2} values obtained at different concentrations of a/eIF2₅₆₅. The chart shows the k_{on} , k_{off} , and K_d values determined as described in Materials and Methods.

observed to be dependent only on the 5′-P₃-end of mRNA but not on its primary sequence, we used the 5′ segment of 2508sh RNA, which had served as a model mRNA in our previous studies (Hasenöhrl et al. 2008). Fluorescein-labeled 2508sh (2508sh_F) RNA or Met-tRNAi^{Met} were incubated with increasing concentrations of a/eIF2₅₆₅, and the K_d values were determined from the k_{on} and k_{off} values derived from the FRET signal(s) between the two ligands. When compared with the K_d of a/eIF2-GTP for the 30S•aIF1•aIF1A complex (0.015 ± 0.007 μM, Fig. 3B), the K_d values of a/eIF2₅₆₅-GTP for 2508sh_F RNA ($K_d = 0.042 \pm 0.01$ μM) and Met-tRNAi^{Met} ($K_d = 0.15 \pm 0.01$ μM) were approximately threefold and 10-fold lower (Fig. 4A), respectively. This indicated that a/eIF2-GTP binds preferentially to 30S ribosomes rather than to the 5′-P₃-end of mRNA or to Met-tRNAi^{Met}. Furthermore, we asked whether 30S-bound a/eIF2₅₆₅-GTP displays a higher affinity for Met-tRNAi^{Met} when compared to the 5′-P₃ terminus of 2508sh RNA. As shown in Figure 4B, the K_d determined

for Met-tRNAi^{Met} binding to a 30S•aIF1•aIF1A•a/eIF2₅₆₅-GTP complex was 0.004 ± 0.002 μM, whereas binding of 5′-P₃-2508sh RNA to this ribosome complex was negligible. In addition, binding of Met-tRNAi^{Met} to a 30S•a/eIF2-GTP complex was hardly affected by the presence of 2508sh RNA (Fig. 4C). Clearly, these in vitro studies do not account for eventual changes in the concentration of components of the translational apparatus which may occur during different stages of growth. Despite this drawback, these in vitro studies provide the first indication that in the archaeon *S. solfataricus* 30S-bound a/eIF2 recruits Met-tRNAi^{Met}.

DISCUSSION

We have shown that the archaeal translation initiation factor aIF1 contacts helices 23 and 24 of its homologous 16S rRNA (Fig. 1), thereby occupying a position near inter-subunit bridges of the 30S subunit (Spahn et al. 2001; Yusupov et al.

FIGURE 4. Association of a/eIF2 with different binding partners. (A) The k_{on} values for a/eIF2₅₆₅ binding to Met-tRNAi_F^{Met} (■) and to 5'-P₃-2508sh_F RNA (▼) were determined by titration of the respective k_{app} values obtained at different concentrations of a/eIF2₅₆₅. (B) The k_{on} values for Met-tRNAi_F^{Met} (■) and 5'-P₃-2508sh_F RNA (▼) binding to a 30S•aIF1•aIF1A•a/eIF2₅₆₅-GTP complex were determined by titration of the respective k_{app} values obtained at different concentrations of the 30S•aIF1•aIF1A•a/eIF2₅₆₅-GTP complex. The charts in A and B show the k_{on} , k_{off} , and K_d values determined as described in Materials and Methods. (C) 30S•a/eIF2 binding to Met-tRNAi_F^{Met} (blue trace) and to equimolar concentrations (see Materials and Methods) of Met-tRNAi_F^{Met} and 5'-P₃-2508sh RNA (orange trace). Control measurements were performed with unlabeled AUG-mRNA and labeled a/eIF2₅₆₅ (black trace, acceptor only) or with labeled Met-tRNAi_F^{Met} and unlabeled a/eIF2 (gray trace, donor only). As donor and acceptor were stable under the experimental conditions, the downward slope of the two control curves may reflect photobleaching.

2001). This topographical localization is equivalent to that found for eukaryal eIF1 and bacterial IF3 (Dallas and Noller 2001; Lomakin et al. 2003), suggesting that aIF1, like its eukaryal and bacterial counterparts, may prevent premature

subunit joining during translation initiation (Subramanian and Davis 1970; Petrelli et al. 2001; Lomakin et al. 2003). In the presence of both, aIF1 and aIF1A, additional protection was observed in helix 34 (Supplemental Fig. S1), possibly

resulting from conformational changes induced by this factor(s). Structural changes in helix 34 of eukaryotic 18S rRNA were also observed upon binding of eIF1 and eIF1A to the 40S subunit (Passmore et al. 2007). These observations may indicate that the functions in translation initiation of these archaeal and eukaryal factors are similar.

Recent experiments indicated that aIF1 stimulates translation initiation complex formation by accelerating binding of a/eIF2 to the 30S ribosomal subunit (Hasenöhrl et al. 2006). Here, we extended these experiments by showing that aIF1 and aIF1A act in concert to stimulate a/eIF2 binding to the ribosome (Fig. 3). However, using different *in vitro* methods, we were unable to demonstrate a direct interaction between aIF1 or aIF1A with a/eIF2. It is worth mentioning that eukaryotic eIF1 binds to a domain of the eIF2 β -subunit (Singh et al. 2004), which is absent in its archaeal homolog a/eIF2 β (Pedulla et al. 2005). Thus, it seems reasonable to speculate that aIF1/aIF1A facilitate a/eIF2 binding to the 30S subunit by inducing structural changes in the ribosomal subunit. Similarly, eIF1 and eIF1A were shown to stabilize conformational changes that apparently accelerate eIF2•GTP•Met-tRNAi^{Met} binding to 40S subunits (Passmore et al. 2007).

We have shown that aIF1, like bacterial IF3 and its eukaryal ortholog eIF1, which discriminate against non-canonical start codons (Hartz et al. 1990; Lomakin et al. 2003), can inhibit ribosome binding of a short mRNA containing a noncanonical AUU start codon (Fig. 2A). Given its topographical localization on the 30S subunit (Fig. 1), it is unlikely that aIF1—like eIF1 (Lomakin et al. 2003) and bacterial IF3 (Gualerzi et al. 2001)—inspects directly the nature of the codon. Instead, as the codon–anticodon interaction is apparently required for the discrimination against the mRNA containing the noncanonical initiation codon (Fig. 2B), it is likely that aIF1 monitors structural changes that occur upon base-pairing of the start codon with the anti-codon of initiator tRNA. Taken together our results suggest that some functions of aIF1, e.g., proofreading and the acceleration of the translation initiation complex formation, are analogous to that of the eukaryotic homolog eIF1 and the bacterial IF3 (Gualerzi et al. 2001; Kapp and Lorsch 2004; Passmore et al. 2007).

Another aspect of translation initiation that this study has taken into scrutiny is the mechanism and the order of events by which the Met-tRNAi^{Met} is recruited by the ribosome during formation of the translation initiation complex in *S. solfataricus*. Formation of this complex is an essential step of protein synthesis in all domains of life, although the mechanism by which this occurs is different, at least in Bacteria and Eukarya. It is accepted that in Eukaryotes eIF2-GTP binds Met-tRNAi^{Met} in the cytoplasm and that it serves as a carrier to bring Met-tRNAi^{Met} to the ribosome (Kapp and Lorsch 2004). In contrast, recent data revealed that in *Escherichia coli* IF2 binds first to the 30S subunit and then recruits fMet-tRNAi^{fMet}

(M Carotti and CO Gualerzi, unpubl.). The situation in *S. solfataricus* is more complex, as a/eIF2 does not only bind to Met-tRNAi^{Met} and to the 30S subunit but also to the 5′-P₃-end of mRNA, whereby binding to the latter serves to protect mRNAs from 5′→3′ decay (Hasenöhrl et al. 2008). Surprisingly, our recent experiments revealed that the affinity of a/eIF2 for the 5′-P₃-end of mRNA was one order of magnitude higher than for Met-tRNAi^{Met}, indicating that—in contrast to Eukaryotes—an assembly of an a/eIF2-GTP•Met-tRNAi^{Met} complex is unlikely in the presence of competing 5′-P₃ mRNA termini. However, the results of the FRET assays in which the *K_d* for all three ligands of a/eIF2, namely, the 30S•aIF1/1A complex (Fig. 3B), Met-tRNAi^{Met}, and the 5′-P₃-end of mRNA (Fig. 4A) were determined, indicate that there is no competition between Met-tRNAi^{Met} and the 5′-P₃-end of mRNA when the Met-tRNAi^{Met} is bound to a/eIF2 in a 30S•a/eIF2•aIF1•aIF1A-GTP•Met-tRNAi^{Met} complex (Fig. 4B,C). The FRET-based calculation of the *K_d*'s of a/eIF2 for Met-tRNAi^{Met} and for the 5′-P₃-end of mRNA are in the same range as determined recently using biochemical assays (Hasenöhrl et al. 2008).

Overall, our results indicate that the Met-tRNAi^{Met} is not carried to the ribosomes by the initiation factor but is instead recruited at the ribosomal level. A working model for translation initiation in the crenarchaeon *S. solfataricus* is shown in Figure 5. Initially, aIF1 and aIF1A induce structural changes in the 30S subunit, which facilitate binding of a/eIF2-GTP. As 30S-bound a/eIF2 has a negligible affinity for 5′-P₃ RNA (Fig. 4B), Met-tRNAi^{Met} is recruited to the 30S•aIF1•aIF1A•a/eIF2-GTP complex. These FRET data would support our working model in that binding of a/eIF2 to the 5′-P₃-end of mRNA, and thereby mRNA stabilization by a/eIF2, is only brought about under conditions when free 30S ribosomes are limiting (Hasenöhrl et al. 2008). Clearly, the model shown in Figure 5 is based on *K_d* values derived from defined *in vitro* experiments. As the intracellular levels of the translational components are unknown in *S. solfataricus*, the working model cannot account for eventual fluctuations in the concentrations of translational components or that of mRNAs. Nonetheless, our recent data indicated that at least during mimicked starvation conditions a/eIF2 is in excess over ribosomes (Hasenöhrl et al. 2008). At least under such conditions the order of events shown in Figure 5 seems conceivable.

Like in Bacteria, a canonical mRNA containing a SD sequence can directly bind to *S. solfataricus* 30S subunits through the SD–anti-SD interaction, whereas leaderless mRNAs, which depend on the start-codon/anti-codon interaction, require a P-site bound tRNAi (Grill et al. 2000; Moll et al. 2001; Benelli et al. 2003). As leaderless mRNAs are prevalent in *Sulfolobus* spp. (She et al. 2001), the postulated mechanism of translation initiation (Fig. 5), i.e., recruitment of Met-tRNAi^{Met} by 30S-bound a/eIF2,

FIGURE 5. Model for the order of events of a/eIF2 binding to different ligands and the sequence of events in translation initiation. When ribosomes are limiting and the number of a/eIF2 molecules exceeds (>) that of free 30S ribosomes, the translation initiation factor may bind preferentially to the 5'-P₃-end of mRNA, a situation that may occur during slow growth conditions (Hasenöhrl et al. 2008). In opposite, when the number of free ribosomes equals or exceeds (>) that of a/eIF2, which may occur during fast growth, the factor binds to ribosomes. Our data revealed that a/eIF2 binding to 30S subunits is accelerated by the aIF1 and aIF1A. Next, Met-tRNA^{Met} is recruited by a/eIF2 being part of the 30S•aIF1•aIF1A•a/eIF2-GTP complex. It remains to be shown whether canonical mRNAs are recruited to the initiation complex before or after Met-tRNA^{Met} binding. In the next step, the correct start-codon/anti-codon interaction is monitored by aIF1 (indicated by a blue arrow). We speculate that the Met-tRNA^{Met} bound in the initiation complex is especially important for the recruitment of leaderless mRNAs. In analogy to *E. coli* (Moll et al. 2004), we consider that a leaderless mRNA is recruited to a 70S initiation complex. The given K_d values were taken from Figures 3 and 4.

could be of particular importance to accelerate translation of this class of mRNAs.

MATERIALS AND METHODS

Preparation of *S. solfataricus* ribosomes, 2508sh RNA, Met-tRNA^{Met}, and translation initiation factors

S. solfataricus ribosomes were prepared from frozen cells as described before (Londei et al. 1986). 2508sh RNA with a triphosphate at the 5'-end (5'-P₃) was prepared as described (Hasenöhrl et al. 2008). The 2508sh RNA corresponds in sequence to nucleotides 1–135 (5'-P₃-ATGATTGTAGGATTGCGGAAA ACTATATAAAAACTATGAGAAAGATGGTATAGAGTTATTGA AAGAGGTCAGTATGAGGCACTAGATATTGCTGGATTGAAT TACGCTGACGTCGATGGGATAATGGCTAAC-3') of the bicistronic *S. solfataricus* 2508 mRNA (1507 nucleotides), encoding the leaderless acetyl-CoA-acetyltransferase (SSO2508) gene (the start codon is underlined) and a downstream gene coding for a predicted nucleic acid binding protein (SSO2509). *E. coli* tRNAⁱ, which is

functionally identical to that of *S. solfataricus* tRNAⁱ (Yatime et al. 2004, 2006), was charged with methionine as described (Pedulla et al. 2005). The a/eIF2 subunits and N-terminal His-tagged aIF1 were prepared as described before (Pedulla et al. 2005; Hasenöhrl et al. 2006).

The gene encoding aIF1A was cloned by means of PCR amplification from *S. solfataricus* genomic DNA. The forward primer aIF1A_FP (5'-GTGTCAAAGGATCCTTGCCTAAGAAAG ATAGAGCGCAGG-3') and the reverse primer aIF1A_RP (5'-TT ATCCTCTTAAGTGGTCAATTACTTCTAGGAATCCGCGG CGC-3') were used for PCR. They contained restriction sites (underlined; BamHI and EcoRI) for insertion into the corresponding sites of the expression vector pRSETB (Invitrogen). The recombinant plasmid was sequenced and transformed into *E. coli* BL21(DE3) (Stratagene). The expression of aIF1A was induced for 3 h with 1 mM IPTG at an OD₆₀₀ of 0.5. The cells were lysed and the cell extracts were heated for 10 min at 70°C and centrifuged at 10,000g for 10 min to precipitate the *E. coli* proteins. The recombinant proteins, which contained an N-terminal tag of six histidines, were purified to homogeneity by affinity chromatography on Ni-NTA agarose following standard protocols (Qiagen).

The purified proteins were dialyzed against storage buffer (10 mM MOPS, 200 mM KCl, 10 mM β -mercaptoethanol, and 10% glycerol) and stored at 80°C in aliquots.

Hydroxyl radical probing with Fe(II)-EDTA

The experiments were performed on a Bio-Logic SFM-400 fast mixing apparatus in quench flow mode as described by Fabbretti et al. (2007), which ensured a high reproducibility of the experiments. Ribosomes and aIF1 were in reaction buffer (10 mM KCl, 20 mM Mg[OAc]₂, 20 mM HEPES at pH 7.1) and were preheated to 65°C for 5 min before use. Syringes 1 and 2 were filled with 2, 5, or 10 μ M aIF1 and 1 μ M *S. solfataricus* 30S ribosomal subunits containing 0.45% H₂O₂, respectively. Equal volumes (20 μ L) of the two solutions were rapidly mixed followed by a reaction time of 2 sec before addition of 20 μ L of a freshly prepared 15 mM Fe(II)-EDTA solution present in syringe 3. The cleavage reaction was allowed to proceed for 20–30 msec before addition of 180 μ L of quenching solution (100 mM Na[OAc]₂ at pH 5.4 in Ethanol) present in syringe 4.

The samples were subjected to primer extension reactions and sequenced by using twenty 5'-³²P-labeled DNA oligonucleotides complementary spanning the 16S rRNA. Primer extension was performed using AMV-RT (Promega). The polyacrylamide gels (8%) were exposed and the bands were quantified using a Typhoon 8600 PhosphorImager and ImageQuant software (Amersham Bioscience), respectively.

Preparation and activity of fluorescently labeled components

AUG-mRNA (5'-GGCAGAGGUGAUAAAUAUUGUUCACGAU-3') and AUU-mRNA (5'-GGCAGAGGUGAUAAAUAUUAUUUCACGAU-3') oligos with 3'-end modification (FAM), termed AUG-mRNA_F and AUU-mRNA_F, were purchased from Microsynth (Switzerland). The SD-sequences 5' of the AUG start codon (bold) and AUU codon (bold) are underlined. Equal labeling of both RNAs with FAM was verified by spectroscopic determination of the RNA concentration to FAM label ratio.

E. coli tRNAi (tRNA_f^{Met}) was labeled at a thiouridine at position 8 with 5-iodoacetamidofluorescein (Invitrogen) according to a recently published protocol (Milon et al. 2007). As determined by reverse phase HPLC, 100% of the tRNAi was labeled with the fluorophore. tRNA_f was then aminoacylated as described above. The aminoacylated and labeled tRNAi is referred to in the text as Met-tRNA_f^{Met}. When compared to unlabeled *S. solfataricus* Met-tRNA_f^{Met}, *E. coli* Met-tRNA_f^{Met} showed the same binding affinity to *S. solfataricus* a/eIF2•GTP in an in vitro filter binding assay (data not shown).

2508sh RNA was labeled (Milon et al. 2007) with fluorescein at the 3' end to prevent interference with binding to a/eIF2 γ . Absorbance measurements showed that the extent of labeling was >95%. Binding of 2508sh_F RNA and 2508sh RNA to a/eIF2 γ was identical in a filter binding assay (data not shown).

A single cysteine was introduced into the α -subunit of a/eIF2 as follows. The amino acid at position 11 (Glutamic Acid) was changed to a cysteine by means of PCR using aIF2_C11_FP (5'-TTTTTTCATATGATTTACAGTAGAAGCAAACCTACCTCATGCGGA GAAATTTTAATTGCAACTG-3') as forward and aIF2_C11_RP

(5'-TTTTTCTCGAGTTTCTTAACCACACTTATAT-3') as reverse primer. The oligonucleotides contained restriction sites (underlined; NdeI and XhoI) for insertion into the corresponding sites of the expression vector pET22b (Novagen). The mutant a/eIF2 α -subunit gene was expressed and the corresponding protein was purified as specified above for aIF1A. The a/eIF2 α ^{C11}-subunit was labeled with the fluorescent dye Atto565 according to Milon et al. (2007). The abbreviation a/eIF2₅₆₅ is used for the reconstituted trimeric factor (Pedulla et al. 2005), consisting of the α ^{C11}₅₆₅, β - and γ -subunits. As the labeled protein showed reduced electrophoretic mobility when compared with the unmodified one, the efficiency of modification was assessed by SDS-PAGE (data not shown). Maximum modification (>95%) was achieved after 1.5 h incubation of a/eIF2 α with Atto565 at room temperature. The activity of a/eIF2₅₆₅ in subunit formation and binding of Met-tRNA_f^{Met} was identical to that of a/eIF2 as determined by native gel electrophoresis and filter binding assays (data not shown). The concentration of active a/eIF2₅₆₅ (see Figs. 3, 4) was determined by a titration filter binding assay using [³⁵S]Met-tRNA_f^{Met} (Smith 1998). This assay revealed that >90% of a/eIF2₅₆₅ was active (data not shown). All labeled components were stable under the assay conditions used.

Rapid kinetic measurements

The measurements were carried out with a KinTec RQF-3 apparatus (KinTec). For a/eIF2₅₆₅ fluorescence excitation was at 565 nm and output was monitored using a KV600 nm long-pass filter. For the Met-tRNA_f^{Met}–a/eIF2₅₆₅ FRET, AUG-mRNA_F/AUU-mRNA_F–a/eIF2₅₆₅ FRET, and 2508sh_F RNA–a/eIF2₅₆₅ FRET experiments excitation was at 460 nm and output was monitored using a KV600 nm long-pass filter. All measurements were performed in 10 mM KCl, 20 mM Mg(OAc)₂, 20 mM HEPES (pH 7.1) at 50°C. All fluorescence and FRET experiments were carried out in triplicates with eight shots per experiment, and the traces presented are an average of three experiments. At least 500 time points occurred in the regions with fluorescence change in each experiment.

Binding of a/eIF2 to 30S subunits

All 30S-containing solutions were incubated for 10 min at 65°C before mixing with a/eIF2₅₆₅. The three subunits of a/eIF2 (α ₅₆₅, β , and γ) were pre-incubated for 10 min at 65°C before they were added to the 30S subunits. The final concentrations were 0.15 μ M a/eIF2₅₆₅, 0.3 μ M 30S, 0.4 μ M aIF1, and 0.4 μ M aIF1A. The data were evaluated with Prism 4.03 (GraphPad Software Inc.) by fitting the two-exponential function $Y = A_0 + A_1 \cdot \exp(-k_{app1} \cdot x) + A_2 \cdot \exp(-k_{app2} \cdot x)$, with k_{app1} and k_{app2} and A_1 and A_2 as the apparent rate constants and the respective amplitudes. To determine average rate constants, k_{av} , which are the inverse of the time at which a reaction reached 50% completion, all k_{app} values and their respective amplitudes were taken into account. The weighted average was calculated as $k_{av} = (k_{app1} \cdot A_1 + k_{app2} \cdot A_2) / (A_1 + A_2)$. To determine the association rate constant (k_{on}) for a/eIF2 binding to the 30S ribosomal subunit, the k_{app2} ($=k_{fast}$) values were determined as a function of a/eIF2 concentration. The values of the slope of the interpolated lines represent the k_{on} of a/eIF2 binding to either 30S ribosomal subunits or to a 30S•aIF1/1A complex. The y -axis intercept of the yielded dissociation rate

constant was interpreted as the dissociation rate constant k_{off} and used for the calculation of the equilibrium binding constant K_d using the equation $K_d = k_{\text{off}}/k_{\text{on}}$.

Binding of Met-tRNA_i^{Met} and 2508sh_F mRNA to a/eIF2₅₆₅

Binding of Met-tRNA_i^{Met} to a/eIF2₅₆₅ was monitored by FRET using fluorescein as fluorescence donor and Atto565 as acceptor. The three subunits of a/eIF2 (α_{565} , β , and γ) were incubated for 10 min at 65°C with or without GTP before they were incubated with 30S subunits, and/or 5'-P₃ (triphosphorylated) 2508sh_F RNA, and Met-tRNA_i^{Met}, respectively. The 30S subunits were incubated for 10 min at 65°C before they were mixed with a/eIF2₅₆₅. The final concentrations were 0.15 μ M a/eIF2₅₆₅, 10 μ M GTP, 0.3 μ M Met-tRNA_i^{Met}, 0.3 μ M 30S, and 0.3 μ M 2508sh_F RNA. Data were evaluated by fitting the two-exponential function $Y = A_0 + A_1 \cdot \exp(-k_1 \cdot x) + A_2 \cdot \exp(-k_2 \cdot x)$. To determine the k_{on} for Met-tRNA_i^{Met} and 2508sh_F RNA binding to a/eIF2-GTP, the k_{app} values were determined as a function of the a/eIF2₅₆₅ concentration (0.05; 0.1; 0.2; and 0.3 μ M). The value of the slope of the interpolated line represents the k_{on} of Met-tRNA_i^{Met} and 2508sh_F binding to a/eIF2₅₆₅. The y -axis intercept of the yielded dissociation rate constant was interpreted as the dissociation rate constant k_{off} and used for the calculation of the equilibrium binding constant K_d .

Binding of AUG-mRNA_F and AUU-mRNA_F to 30S ribosomal subunits

Binding of AUG-mRNA_F or AUU-mRNA_F to 30S•a/eIF2₅₆₅-GTP•Met-tRNA_i^{Met}•aIF1A•(aIF1) complexes was monitored by FRET, whereby mRNA_F was used as fluorescence donor and a/eIF2₅₆₅ as acceptor. The three subunits of a/eIF2 (α_{565} , β , and γ) were incubated for 10 min at 65°C with GTP. The a/eIF2₅₆₅-GTP complex was added to preformed 30S•aIF1A or 30S•aIF1/1A complexes and incubated for 5 min at 65°C before Met-tRNA_i^{Met} was added, and the incubation time was extended for 5 min at 65°C. The 30S•a/eIF2₅₆₅-GTP•Met-tRNA_i^{Met}•aIF1A•(aIF1) complex was then mixed with mRNA_F in the KinTec apparatus. The final concentrations were 0.15 μ M a/eIF2₅₆₅, 10 μ M GTP, 0.2 μ M Met-tRNA_i^{Met}, 0.3 μ M 30S, 0.45 μ M aIF1, 0.45 μ M aIF1A, and 0.2 μ M AUG-mRNA_F or AUU-mRNA_F. Data were evaluated by fitting the two-exponential function $Y = A_0 + A_1 \cdot \exp(-k_1 \cdot x) + A_2 \cdot \exp(-k_2 \cdot x)$. The weighted average was calculated as $k_{\text{av}} = (k_{\text{app1}} \cdot A_1 + k_{\text{app2}} \cdot A_2) / (A_1 + A_2)$, where k_{app1} and k_{app2} and A_1 and A_2 are the apparent rate constants and the respective amplitudes estimated from two-exponential fitting of the time courses.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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