

The archaeal eIF2 homologue: functional properties of an ancient translation initiation factor

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ABSTRACT

The eukaryotic translation initiation factor 2 (eIF2) is pivotal for delivery of the initiator tRNA (tRNA_i) to the ribosome. Here, we report the functional characterization of the archaeal homologue, a/eIF2. We have cloned the genes encoding the three subunits of a/eIF2 from the thermophilic archaeon *Sulfolobus solfataricus*, and have assayed the activities of the purified recombinant proteins *in vitro*. We demonstrate that the trimeric factor reconstituted from the recombinant polypeptides has properties similar to those of its eukaryal homologue: it interacts with GTP and Met-tRNA_i, and stimulates binding of the latter to the small ribosomal subunit. However, the archaeal protein differs in some functional aspects from its eukaryal counterpart. In contrast to eIF2, a/eIF2 has similar affinities for GDP and GTP, and the β -subunit does not contribute to tRNA_i binding. The detailed analysis of the complete trimer and of its isolated subunits is discussed in light of the evolutionary history of the eIF2-like proteins.

INTRODUCTION

During translation initiation, ribosomes identify the initiation codon on the mRNA and set the correct reading frame for decoding. This rate-limiting step is carried out in different ways in the primary domains of cell descent. In bacteria, the small ribosomal subunit interacts directly with the mRNA by base-pairing of the Shine–Dalgarno (SD) motif, preceding the initiation codon, with the anti-SD motif at the 3' end of 16S rRNA. The initiation step is assisted by three protein factors, IF1, IF2 and IF3 (1,2). In eukarya, selection

of the initiation codon entails a 'scanning' mechanism, whereby the 40S subunit binds to the capped 5' end of the mRNA and then slides downstream until the initiator AUG, usually the first one available, is encountered (3). This process is promoted by many factors, several of which are involved in cap recognition and mRNA unwinding, while others interact with the ribosome and/or the mRNA to ensure correct selection of the initiation codon (4,5).

In archaea, two different mechanisms for translational initiation seem to exist (6,7). One is based on a canonical SD/anti-SD interaction and operates on internal cistrons of polycistronic mRNAs. In contrast, monocistronic mRNAs as well as proximal cistrons of polycistronic mRNAs are frequently devoid of a 5'-untranslated region. These leaderless mRNAs are decoded by an initiation mechanism independent of the SD/anti-SD interaction, which requires, analogously to bacteria (8), pairing of the start codon with initiator-tRNA (tRNA_i) (7). The complexity of archaeal translational initiation seems to be underscored by the presence of a larger-than-bacterial set of initiation factors. All archaeal genomes encode about 10 proteins homologous to eukaryal initiation factors (9,10).

A central step in translation initiation is the delivery of tRNA_i to the ribosomal P site. This task is assisted in bacteria by the monomeric protein IF2, and is performed in eukarya by the trimeric complex eIF2, consisting of the α -, β -, and γ -subunits, none of which is homologous to bacterial IF2. Eukarya possess a homologue of bacterial IF2, termed eIF5B, which seems to act at a later initiation step, promoting the joining of the 60S ribosomal subunit to the 40S initiation complex (11). Like eukarya, archaea possess both IF2 and eIF2 homologues, but their respective functions remain poorly characterized.

In this study, we have analysed a number of functional features of the eIF2-like protein (herein denoted as a/eIF2) of the thermophilic archaeon *Sulfolobus solfataricus*.

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The genome of this organism, as well as those of the other archaea sequenced so far, contains homologues of all three subunits of the eukaryal factor (12). In *S.solfataricus*, the largest subunit is the γ -homologue, which comprises 415 amino acids and contains a recognizable G-domain. The α -subunit homologue contains 266 amino acids. The β -subunit is the smallest polypeptide of a/eIF2 (139 amino acids in *S.solfataricus*) and has experienced the most extensive evolutionary drift with respect to the eukaryotic protein. Indeed, eukaryotic β -subunits are about twice the size of archaeal ones, and include domains involved in the interaction with two other proteins essential for eIF2 function: eIF2B, a factor required for GDP/GTP exchange (13,14) and eIF5, necessary for the hydrolysis of eIF2-bound GTP (14–16). Neither eIF2B nor eIF5 have homologues in archaeal genomes. The conserved region of the β -subunit in archaea and eukaryotes includes a domain containing a zinc-finger motif, which has been implicated in controlling the accuracy of initiation codon recognition (17). Recently, it has been shown that the recombinant α -, β - and γ -subunits of a/eIF2 from the hyperthermophilic archaeon *Pyrococcus abyssi* can re-associate *in vitro* to form a trimer able to bind selectively Met-tRNA_i (18). The tRNA binding site seems to reside on the γ -subunit (19), however an α - γ dimer is required for a stable interaction with Met-tRNA_i (18).

In this work, we have cloned the genes and purified the corresponding three subunits of a/eIF2 from the crenarchaeon *S.solfataricus*. *In vitro* assays carried out with the individual a/eIF2 subunits, with dimers thereof and with the complete trimer have confirmed that the factor binds Met-tRNA_i, and that this GTP-dependent binding requires the α - γ dimer. We also show that a/eIF2 α - γ and α - β - γ strongly stimulate binding of Met-tRNA_i to the small ribosomal subunit in the presence of GTP. Furthermore, an analysis of guanine nucleotide exchange has revealed that a/eIF2 has a similar affinity for GTP and GDP, and that the G nucleotide binding properties of the isolated γ -subunit are similar to those of the complete factor. In addition, the γ -subunit was found to be responsible for the interaction of the factor with ribosomes. Finally, we show that a/eIF2 lacks an intrinsic ribosome-dependent GTPase activity. The differences and similarities with the eukaryal factor are discussed.

MATERIALS AND METHODS

Cloning of the genes and purification of the a/eIF2 subunits

The genes encoding the α -, β - and γ -subunits of a/eIF2 were cloned by means of PCR amplification from *Sulfolobus solfataricus* genomic DNA: 30–35mer oligonucleotides complementary to the 5' and 3' ends of the respective coding sequences were used, which contained appropriate restriction sites for insertion into the expression plasmid pRSETB (Invitrogen). The recombinant plasmids were selected in *E.coli* Top10 (Invitrogen), and then transformed into *E.coli* BL21(DE3) (Stratagene). The expression of the a/eIF2 genes was induced for 3 h with 1 mM IPTG at an OD₆₀₀ of 0.5–0.6. The cells were then lysed and the extracts were enriched for the thermophilic recombinant proteins by heating at 70°C for 10 min followed by centrifugation at 10 000 g for 10 min

to remove the precipitated host proteins. The recombinant polypeptides, which contained an N-terminal tag of six histidines, were purified by affinity chromatography on Ni-NTA agarose following standard protocols. The purified proteins were dialysed against storage buffer (10 mM MOPS, 200 mM KCl, 10 mM β -mercaptoethanol and 10% glycerol) and stored at –80°C in aliquots.

Interaction between the a/eIF2 polypeptides

To test whether the recombinant polypeptides interact and form dimeric and trimeric complexes, 30 pmol of α -, β - and γ -proteins were mixed in all possible combinations in a buffer containing 30 mM KCl, 0.5 mM MgCl₂ and 50 mM Tris-HCl, pH 7.1, in the presence or absence of 1 mM GTP. The proteins and their complexes were visualized by non-denaturing electrophoresis on 12% polyacrylamide gels prepared in acetate buffer (120 mM potassium acetate and 72 mM acetic acid, pH 4.3). The gels included a stacking overlay of 4% polyacrylamide in acetate buffer (120 mM potassium acetate and 12 mM acetic acid, pH 6.8). The running buffer was 133 mM acetic acid and 350 mM β -alanine, pH 4.4. After the run, the gels were stained with Coomassie brilliant blue.

Preparation of ribosomes and tRNA_i

70S ribosomes and 30S and 50S ribosomal subunits were obtained from frozen *S.solfataricus* cells as described previously (20). Purified tRNA_i was obtained by *in vitro* transcription of a synthetic *S.solfataricus* tRNA_i gene. Two DNA oligomers were created based on the sequence of the *S.solfataricus* tRNA_i gene as deduced from the published genome sequence (12). The following oligonucleotides were used:

tRNA_i: 5'-GTGTAATACGACTCACTATAAGCGGGGTGGGCTAGCCTGGTAAGGCCGCGGGGCTCATAACCCGAGGTCCTGGTTCAAATCCAGGCCCGCTACA-3'.

anti-tRNA_i: 5'-AGCTTGGTAGCGGGGCCTGGATTTGAAACCAGGGACCTCGGGGTTATGAGCCCCGCGGCCTTACCAGGCTAGCCACCCCGCTTATAGTGAGTCGTATTACACGTA-3'.

The tRNA_i primer contained a T7 promoter directly adjacent to the tRNA_i gene allowing *in vitro* transcription of an oligoribonucleotide initiating with the 5'-terminal A of Met-tRNA_i. For plasmid construction, the oligomers were phosphorylated, mixed and annealed to form double-stranded DNA by heating at 100°C followed by slow cooling to 37°C. This DNA fragment was then ligated into plasmid pBS-SK (Stratagene) digested with DraIII and HindIII. The ligation mixture was transformed in *E.coli* Top10 cells. The recombinant plasmid (pBS-tRNA_i) was sequenced to verify the authentic gene sequence. To obtain tRNA_i, 25 nM of pBS-tRNA_i were incubated with 2.5 U of Pwo DNA polymerase in 100 μ l of 25 mM KCl, 5 mM (NH₄)₂SO₄, 200 μ M dNTP, 10 mM Tris-HCl, pH 8.8, and 600 nM of the following primers: forward primer: 5'-GGCCACTACGTGTAATACGACTCAC-3'; reverse primer: 5'-TGGTAGCGGGGCCTGGATTTGAAACCAGG-3'. The amplified fragment was purified using the QIAquick PCR purification kit (Qiagen). The tRNA_i was then transcribed at 37°C for 1 h in 100 μ l of a mixture containing 50 U of T7 RNA polymerase, 6 mM MgCl₂, 2 mM spermidine,

10 mM DTT, 40 mM Tris-HCl, pH 7.9, 0.5 mM of each NTP and 10 U RNase inhibitor. As transcription initiated with an A residue, high concentrations of PCR product (50–60 pmol/100 μ l) were used. The reaction mixture was extracted once with phenol, once with phenol/chloroform and finally with chloroform/isoamylalcohol. The RNA was precipitated with ethanol, dried and resuspended in an appropriate volume of diethylpyrocarbonate-treated H₂O. That the transcript indeed initiated with a 5'-terminal A was experimentally verified by primer extension.

The purified tRNA_i was charged with methionine, cold or labelled with [³⁵S], using a recombinant *E. coli* methyltransferase: Briefly, ~200 pmol of tRNA_i were incubated at 37°C for 30 min with 150 pmol methyltransferase, 4 mM ATP and 100 μ M [³⁵S]methionine [specific activity (s.a.) 1000 Ci/mmol] or unlabelled methionine as required. To check the efficiency of aminoacylation, 100 pmol of Met-tRNA_i was loaded on a 6.5% polyacrylamide gel made in 0.1 M NaOAc, pH 5 and 8 M urea. The gel was run for 24 h at 500 V (~12 V/cm) and then stained with ethidium bromide. The bands corresponding to Met-tRNA_i and uncharged tRNA_i were quantified using the ImageQuant software; ~35% of the tRNA_i was found to be aminoacylated under these conditions. To recover the charged Met-tRNA_i, the incubation mixture was subjected to phenol extraction. The RNA precipitated with ethanol from the aqueous phase was finally resuspended in 10 mM acetate buffer, pH 5.2.

Guanine nucleotides binding and exchange

The purified a/eIF2 subunits (20 pmol each), alone or in combinations, were incubated for 10 min at 65°C in buffer containing 30 mM KCl, 50 mM HEPES, pH 7.5, 0.5 mM MgCl₂, and 1 mM DTT (incubation buffer) with increasing concentrations (0–100 pmol) of [³H]GDP (~1300 c.p.m./pmol) or [³²P]GTP (~6000 c.p.m./pmol). The samples were filtered through Millipore 0.22 μ m nitrocellulose disks, which were washed extensively with incubation buffer. The disks were dried and the amount of retained radioactivity was measured in a liquid scintillation counter. The K_d values for nucleotide binding were determined graphically from Scatchard plots derived from the binding curves.

To determine the rate of G nucleotides exchange, isolated γ -subunits (100 pmol) or the complete trimer reconstituted with 100 pmol of each α -, β - and γ -subunits were incubated with a 10-fold excess of [³H]GDP for 10 min at 65°C. The mixtures were supplemented with 10 mM unlabelled GTP or GDP, and the incubation was continued at 80°C while withdrawing samples after 0, 3, 5, 7 and 10 min. The samples were filtered through 0.22 μ m nitrocellulose disks, which were washed with incubation buffer, dried and then subjected to scintillation counting.

[³⁵S]Met-tRNA_i interaction with a/eIF2

To assay the capacity of a/eIF2 to interact with [³⁵S]Met-tRNA_i, 50 pmol of the recombinant subunits, individually or in different combinations, were incubated at different temperatures ranging from 25 to 75°C in 20 μ l (final volume) of incubation buffer containing 1 mM GTP and 100 pmol of [³⁵S]Met-tRNA_i (s.a. ~500 c.p.m./pmol). Incubation was carried out for 15 min. Samples were withdrawn at times 1, 3, 5,

10 and 15 min. The samples were filtered through 0.22 μ m nitrocellulose disks, which were washed and processed as described above.

Protection by a/eIF2 of [³⁵S]Met-tRNA_i against spontaneous hydrolysis was assayed in 20 mM HEPES-NaOH (pH 8.0), 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.2 mg/ml BSA and 5% glycerol, in the presence of 1 mM GTP, 1 mM GDP or 1 mM Gpp(NH)p, and 50 nM of [³⁵S]Met-tRNA_i and a/eIF2 (0.2 and 2 μ M). Before the addition of [³⁵S]Met-tRNA_i, the three subunits were incubated in the presence of GTP [GDP or Gpp(NH)p] for 10 min at 65°C to allow subunit formation. The mixtures were then incubated at 65°C. To determine the rate of de-acylation, aliquots were withdrawn at various times (10–30 min) and precipitated with 5% trichloroacetic acid. The precipitates were collected on Whatman filters, which were washed 2 \times with ice-cold 5% TCA, and then dried for 10 min at 85°C. The amount of the retained [³⁵S]Met-tRNA_i was determined by scintillation counting.

Interaction of a/eIF2 subunits with ribosomes

To determine which of the a/eIF2 polypeptides contained the domain for interaction with ribosomes, 50 pmol of each α -, β - or γ -subunit were incubated at 65°C for 10 min in incubation buffer in the presence of 1 mM GTP and ~100 pmol of Met-tRNA_i (charged with cold methionine). The samples were supplemented with 30 pmol of ribosomes and incubation was continued for another 10 min. The reaction was stopped by adding an appropriate volume of 10 \times loading buffer (0.02% bromophenol blue, 0.02% xylene cyanol and 50% glycerol), and the samples were immediately loaded on running non-denaturing 4% polyacrylamide gels made in 20 mM potassium acetate, 2.5 mM MgCl₂ and 40 mM Tris-HCl, pH 6. Electrophoresis was continued at 4°C and 50 mA for 4–5 h. After the run, the samples were transferred to Hybond N membranes by electroblotting and the position of the recombinant proteins was visualized by immunostaining with anti-His tagged antibodies.

Interaction of [³⁵S]Met-tRNA_i with ribosomes

The ability of a/eIF2 to stimulate binding of [³⁵S]Met-tRNA_i to ribosomes was tested as follows: 50 pmol of each α -, β - and γ -proteins, or α - γ or γ only were mixed and incubated for 10 min at 65°C in 50 μ l incubation buffer with 1 mM GTP to allow formation of the trimeric or dimeric complexes. [³⁵S]Met-tRNA_i (100 pmol, s.a. ~500 c.p.m./pmol) were added and incubation was continued for 5 min. Finally, the mixtures were supplemented with 30 pmol of ribosomes and further incubated for 10 min. The samples were immediately electrophoresed on non-denaturing 4% polyacrylamide gels as described above, then dried and exposed to an X-ray film to visualize the labelled tRNA_i. In parallel, the gels were stained with Coomassie brilliant blue to determine the position of the ribosomes.

GTPase activity assay

The presence of a ribosome-dependent GTPase activity of trimeric a/eIF2 was assayed as follows: 20 pmol of each α -, β - and γ -subunits were mixed in 50 μ l (final volume) of 70 mM KCl, 25 mM Tris-HCl, pH 7.5, 7 mM MgCl₂, 1 mM

DTT and 35 μM [^{32}P]GTP (s.a. ~ 15 mCi/mmol). The mixture was incubated for 5 min at 65°C to allow formation of the trimer. Met-tRNA_i (20 pmol; charged with unlabelled methionine) was then added and the incubation was continued for another 5 min. Then, the reaction mixture was further supplemented with 5 pmol of 70S ribosomes and 5 pmol of a 30mer RNA oligonucleotide mimicking a translation initiation region endowed with a SD-motif (5'-UUUGAGGUGA-CUCUCUCAUGUUUUUUUUU-3': start codon and SD motif are underlined). Incubation was continued at 65°C for 10 min and samples were withdrawn at 0, 1, 5 and 10 min. The samples were supplemented with an equal volume of 1 M HClO₄ and 2 mM KH₂PO₄, gently shaken and further supplemented with 3 vol of 20 mM Na₄MoO₄. Finally, an equal volume of ethyl acetate was added, the mixture was shaken vigorously and centrifuged for 5 min at 6500 r.p.m. to separate the phases: 200 μl were withdrawn from the top phase and the free [^{32}P] present therein was measured in a liquid scintillation counter.

RESULTS

Purification of the three *S.solfataricus* a/eIF2 subunits

The genes encoding the α -, β - and γ -subunits of *S.solfataricus* a/eIF2 were cloned by PCR amplification of genomic DNA and inserted into the *E.coli* 'His-tag vector' pRSET to facilitate purification after over-expression *in vivo*. The recombinant proteins were purified by a two-step procedure, including heating of the *E.coli* lysates at 70°C for 10 min to remove most of the host mesophilic proteins, followed by selective capture of the recombinant polypeptides by affinity chromatography on a Ni-NTA resin. As shown in Figure 1, the three recombinant polypeptides were purified to near homogeneity. They had the expected molecular weights of ~ 45 kDa (γ -subunit), 28 kDa (α -subunit) and 15 kDa (β -subunit). However, their yield was markedly different: the α -subunit was produced in large amounts, whereas the γ - and, particularly, the β -subunit were produced in lower quantities. The histidine-tags were not removed, since they apparently did not affect the activity assays, as previously noted for the *P.abysyi* factor (18).

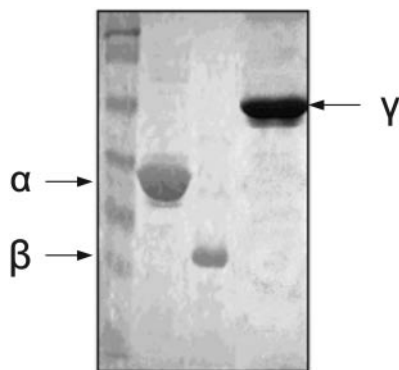


Figure 1. Purification of the recombinant a/eIF2 subunits. The positions of the purified α -, β - and γ -subunits in the Coomassie blue stained SDS-polyacrylamide gel are shown by arrows. The molecular weight markers are (from top to bottom): 90, 67, 45, 30, 21 and 14 kDa.

Interaction between the subunits

The functionality of the purified recombinant polypeptides was first tested by monitoring their capacity to form dimeric and trimeric complexes. Equimolar amounts of the a/eIF2 subunits as well as combinations thereof were incubated at 65°C for 15 min and then electrophoresed on acidic, non-denaturing polyacrylamide gels. As shown in Figure 2, under these conditions the three recombinant polypeptides were competent for mutual recognition. When mixed together in equimolar amounts, the α -, β - and γ -proteins interacted quantitatively to reconstitute the trimeric complex; essentially no free subunits were left over under these conditions (Figure 2). The quantitative formation of α - γ and β - γ dimers was likewise readily observed, whereas the α - and β -subunits did not interact with each other. These experiments supported previous data (18,19) in that the γ -subunit acts as a scaffold for the assembly of the trimer. As *S.solfataricus* is an extreme thermophile, we assayed whether temperature affected these protein-protein interactions. We found that dimeric and trimeric complexes formed readily at room temperature, thus indicating that even at non-physiological temperatures, the proteins are in an interaction-competent conformation (data not shown). The polypeptides were able to assemble also in the presence of GDP as well as without guanine nucleotides, showing that GTP activation of the γ -subunit is not required for complex formation.

Guanine nucleotide binding and exchange

Having ascertained the ability of the a/eIF2 polypeptides to reconstitute the native complex, we next analysed the capacity of the individual subunits and of combinations thereof to bind GDP and GTP. The aim of these experiments was to compare the relative affinity for guanine nucleotides of a/eIF2 with that of its eukaryal counterpart. It is known that the eukaryal eIF2 has a significantly higher affinity for GDP than for GTP.

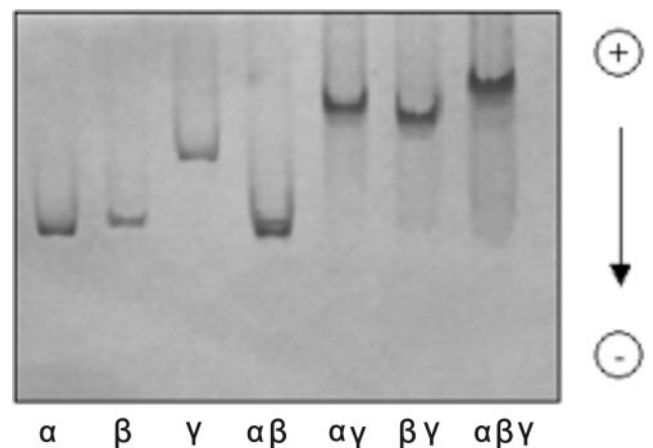


Figure 2. Reconstitution of the trimeric a/eIF2 with purified subunits. The α -, β - and γ - polypeptides were mixed in equimolar amounts (50 pmol each) and incubated at 65°C for 15 min. The individual subunits and mixtures thereof were electrophoresed on non-denaturing polyacrylamide gels (see Materials and Methods). It should be noted that in the native gel system the more basic α -subunits (PI 9.2; 28 kDa) moved slightly ahead of the β -subunits (Isoelectric point 8.1; 15 kDa) despite the difference in size. Migration of the proteins from the cathode towards the anode is depicted on the right-hand side.

As a consequence, the conversion of GDP-eIF2 to GTP-eIF2 requires the pentameric nucleotide exchange factor eIF2B (13). However, archaeal genomes lack apparent homologues of the catalytic subunits of the eIF2B complex (21), which could suggest that an exchange factor is not required for a/eIF2. A 20 pmol of each recombinant subunit of *S.solfataricus* a/eIF2 was incubated in all possible combinations (except α - β) with increasing amounts of [³H]GDP or [³²P]GTP at 65°C, and the amount of bound nucleotide was determined by a filter-binding assay. The quantitative interaction of the recombinant subunits, i.e. the formation of dimers and trimers, was separately verified by non-denaturing gel electrophoresis (data not shown; see Figure 2).

As shown in Figure 3, the binding curves revealed that the guanine nucleotide binding capacity resides entirely on the γ -subunit. In fact, protein complexes containing the γ -subunit (α - γ , β - γ or the trimeric factor) displayed GDP and GTP binding curves essentially overlapping with those obtained with the γ -subunit alone, while neither α nor β (data not shown) bound to G nucleotides. These results indicated that

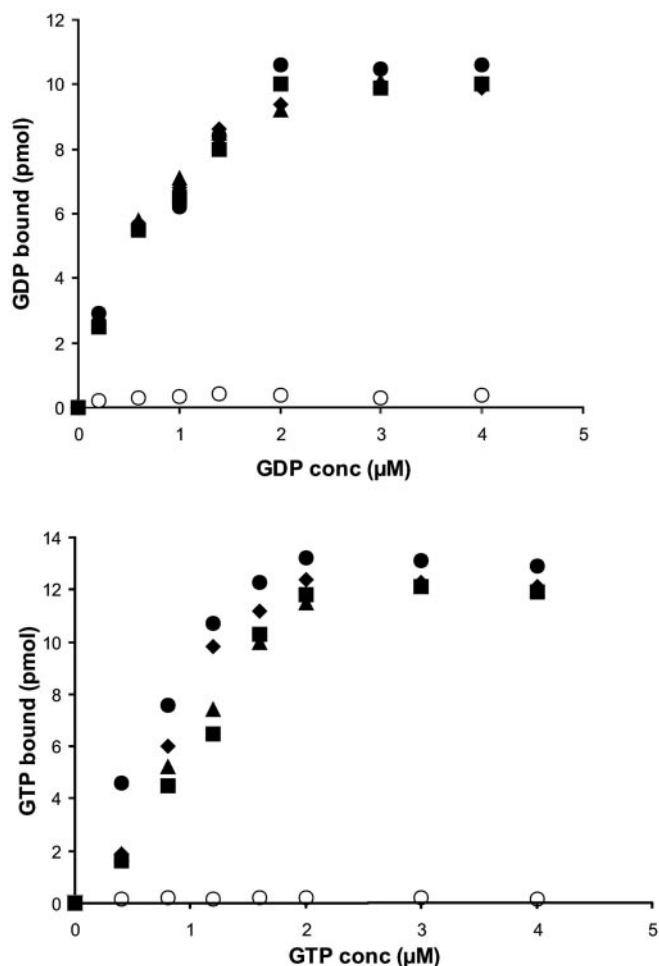


Figure 3. GDP and GTP binding by a/eIF2. The a/eIF2, the individual subunits and combinations thereof were incubated with [³H]GDP (top) or [³²P]GTP (bottom) and the amount of radiolabelled nucleotide retained by the proteins was determined by filtration through nitrocellulose filters. Closed squares, trimeric a/eIF2; closed circles, β - γ dimer; closed triangles, α - γ dimer; closed diamonds, γ -subunit; and open circles, α -subunit.

the G-domain on the γ -subunit is fully competent for nucleotide binding and that binding is not affected by either the α - or β -subunits. From the binding curves shown in Figure 3, we calculated the K_d values for GDP and GTP of the γ -subunit and of the trimeric factor. The values (Table 1) were very similar in all cases, indicating that the G-nucleotide binding domain of a/eIF2, unlike that of eIF2, has comparable affinities for GDP and GTP. For verification, we also measured the off-rate for bound radiolabelled GDP in the presence of an excess of unlabelled GDP or GTP, for the γ -subunit as well as for the reconstituted a/eIF2 trimer. A 100-fold molar excess of cold GDP or GTP was added to the γ -subunit or to the a/eIF2 trimer, both of which had been saturated with [³H]GDP. Samples were withdrawn at different times thereafter to determine the amount of radioactive nucleotide retained. As shown in Figure 4, at the optimal growth temperature for *S.solfataricus* (80°C) bound GDP was substituted by either GDP or GTP at a comparable rate and nucleotide exchange was complete within 4 min. The γ -subunit and the complete trimer behaved similarly. Taken together, the binding curves and the exchange kinetics suggested that the GTP-a/eIF2 complex forms spontaneously without an auxiliary factor. These results are in agreement with the observation that archaeal genomes harbour only two homologues of the five polypeptides composing the eukaryal eIF2B, and that these two do not include the catalytic subunits known to be essential for guanine nucleotide exchange in eukaryotes (21).

Binding of Met-tRNA_i to a/eIF2

The primary role of the eukaryal eIF2 in translation entails the delivery of tRNA_i to ribosomes through formation of a stable ternary complex with GTP and tRNA_i. Recently, it was shown

Table 1. K_d values for GDP and GTP binding by trimeric a/eIF2 and isolated γ -subunits

	γ	$\alpha\beta\gamma$
K_d (GDP)	$(4 \pm 0.2) \times 10^{-7}$	$(3.9 \pm 0.3) \times 10^{-7}$
K_d (GTP)	$(4.8 \pm 0.3) \times 10^{-7}$	$(3.6 \pm 0.4) \times 10^{-7}$

The values shown represent the average \pm SD of four independent experiments.

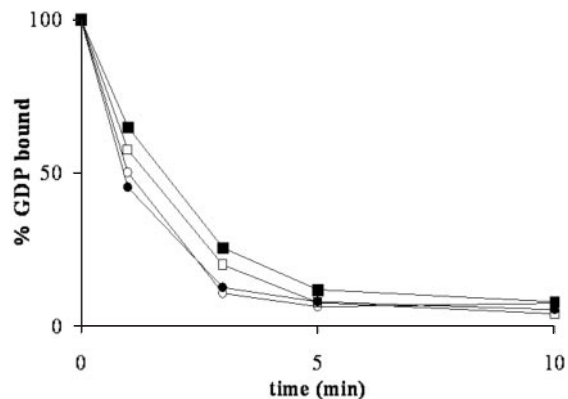


Figure 4. Guanine nucleotide exchange on trimeric a/eIF2 and the γ -subunit. The proteins were saturated with [³H]GDP for 15 min at 65°C. Then, a 100-fold excess of cold GDP (closed symbols) or GTP (open symbols) was added and incubation was continued at 80°C. Samples were withdrawn at the indicated times. Squares, trimeric a/eIF2; and circles, γ -subunit.

that archaeal *a/eIF2* from *P.abysssi*, reconstituted *in vitro* from the recombinant subunits, has a similar capacity for selective binding to Met-tRNA_i (18). Accordingly, we tested the capacity of *S.solfataricus a/eIF2* to interact stably with Met-tRNA_i by employing a filter-binding assay.

The three *a/eIF2* subunits and combinations thereof were incubated at 70°C in the presence of 1 mM GTP with *S.solfataricus* [³⁵S]Met-tRNA_i. Samples were withdrawn at different times upon incubation and filtered through nitrocellulose disks as described above. As shown in Figure 5A, a stable complex with [³⁵S]Met-tRNA_i was formed exclusively by either the complete trimer or the α - γ dimer. The binding kinetics of these complexes were hardly distinguishable, demonstrating that the α - γ dimer is necessary and sufficient for the stable interaction with Met-tRNA_i, as previously observed by Yatime *et al.* (18). At variance with these authors, we observed very little retention of Met-tRNA_i by the isolated γ -subunit. This could be due to the different techniques used in both studies since filter binding only captures fairly stable

RNA-protein complexes. It is worth noting that Met-tRNA_i binding was strictly dependent on temperature, being very low at 25°C and reaching an optimum between 60 and 75°C (data not shown). At the optimal temperature, saturation was complete within ~5 min (Figure 5A). The temperature-dependent binding of Met-tRNA_i cannot be attributed to a lack of interaction among the subunits as dimeric and trimeric complexes formed readily at low temperature (data not shown). Thus, we assume that this observation is due to a direct effect of high temperature on the conformation of the Met-tRNA_i binding domain(s) of *a/eIF2*.

As another means to demonstrate binding to and protection of Met-tRNA_i by *a/eIF2*, we tested whether the trimeric factor protects the charged tRNA_i from deacylation. Without protection by *a/eIF2* of Met-tRNA_i, the tRNA was nearly completely deacylated after 30 min (Figure 5B). Similarly, in the presence of GDP, no protection by *a/eIF2* of Met-tRNA_i was observed (Figure 5B), verifying that Met-tRNA_i binding to *a/eIF2* requires GTP. In contrast, in the presence of 2 μ M *a/eIF2* and 1 mM GTP ~80% of the tRNA_i remained acylated after 30 min. The addition of non-hydrolysable Gpp(NH)p had no effect on the protection, which indicated that GTP is not hydrolysed under these conditions.

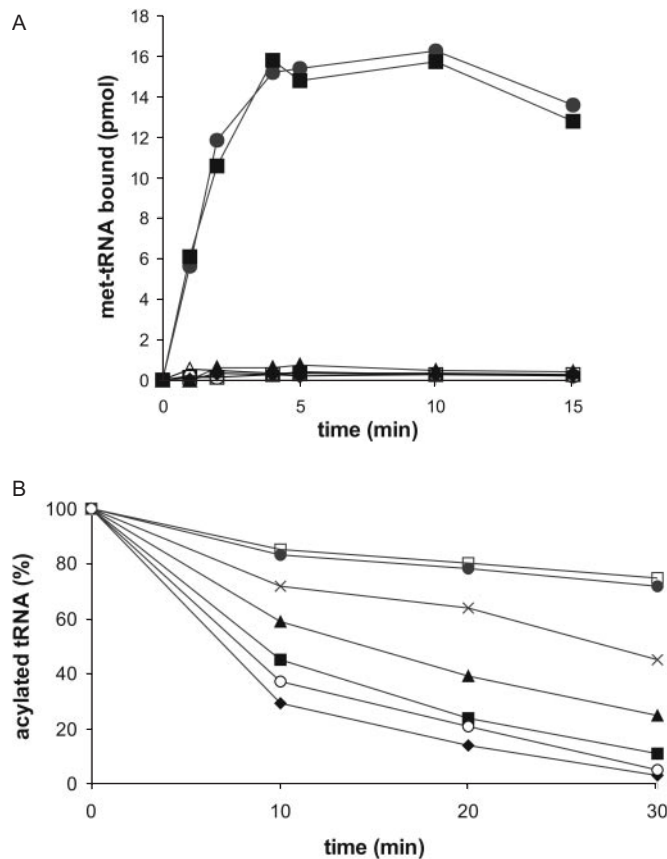


Figure 5. (A) Interaction of [³⁵S]Met-tRNA_i with *a/eIF2* subunits, different dimeric complexes and the trimeric *a/eIF2* at 70°C. For details see Materials and Methods. Closed circles, complete trimer; closed squares, α - γ dimer; open circles, α - β dimer; open squares, β - γ dimer; closed diamonds, α -subunit; closed triangles, γ -subunit; and crosses, β -subunit. (B) Protection of Met-tRNA_i from spontaneous hydrolysis by *a/eIF2*. [³⁵S]Met-tRNA_i was incubated with *a/eIF2* in the presence of GTP, GDP or Gpp(NH)p and the retained radioactivity was determined by filter binding assays. closed diamonds, 1 mM GTP without *a/eIF2* added; open circles, 2 μ M *a/eIF2* and 1 mM GDP; closed squares, 0.2 μ M *a/eIF2* and 1 mM GTP; closed triangles, 0.5 μ M *a/eIF2* and 1 mM GTP; crosses, 1 μ M *a/eIF2* and 1 mM GTP; closed circles, 2 μ M *a/eIF2* and 1 mM GTP; and open squares, 2 μ M *a/eIF2* and 1 mM Gpp(NH)p.

Interaction of *a/eIF2* with ribosomes

Next, we attempted to determine the *a/eIF2* domain responsible for the interaction with ribosomes. High-salt purified *S.solfataricus* 70S ribosomes were incubated at 70°C for 15 min with each of the purified *a/eIF2* subunits. After incubation, the samples were loaded on a non-denaturing polyacrylamide gel and electrophoresis was continued for 5 h. The position of the *a/eIF2* subunit proteins in the gel was visualized by western blotting using antibodies directed against the His-tag. Since free *a/eIF2* subunits have a basic isoelectric point and were expected to run to the anode in the used electrophoresis system, only ribosome-associated factors could be detected. As shown in Figure 6, neither the α - nor the β -subunits was detectably retained by ribosomes. The γ -polypeptide associated with a band that contained 30S subunits as determined by Coomassie blue staining (Figure 6) and western blotting using polyclonal antibodies raised against purified *S.solfataricus*

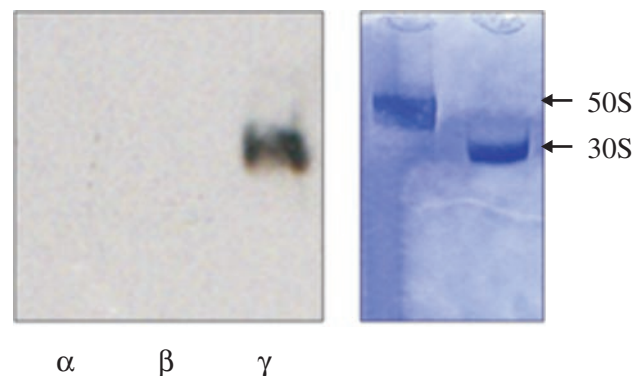


Figure 6. The γ -subunit of *a/eIF2* interacts with the 30S subunit. Left panel: each *a/eIF2* subunit was incubated with 70S ribosomes and the complexes were separated by non-denaturing PAGE. Western blotting with anti-His tag antibodies was used to visualize the proteins in the gel. Right panel: position of the ribosomal subunits as determined separately by Coomassie blue staining.

30S ribosomes (data not shown). We therefore concluded that the $\alpha/\text{eIF2}$ factor interacts with the small ribosomal subunit by means of a domain located on the γ -subunit.

Stimulation of Met-tRNA_i binding to ribosomes

The next experiments were aimed at determining whether $\alpha/\text{eIF2}$, like its eukaryal counterpart, stimulates the formation of a pre-initiation complex containing the small ribosomal subunit and Met-tRNA_i. To this end, the reconstituted trimeric factor was pre-incubated with GTP and [³⁵S]Met-tRNA_i to allow formation of the ternary complex. After 10 min of pre-incubation, 70S ribosomes were added and the incubation was continued at 65°C for 15 min. The formation of the pre-initiation complexes was then monitored by means of non-denaturing gel electrophoresis.

The results shown in Figure 7 illustrate that $\alpha/\text{eIF2}$ did stimulate binding of Met-tRNA_i to the ribosome. The [³⁵S]Met-tRNA_i appeared to interact with the 30S subunits, even when 70S ribosomes were added to the assay. However, it should be noted that *S.solfataricus* '70S' ribosomes are actually a mixture of dissociated subunits, since the monomeric particle is stable only when engaged in translation (20). A small quantity of the nucleic acid bound to the 30S subunits in the absence of any factor and the addition of increasing amounts of $\alpha/\text{eIF2}$ resulted in a linear increase of [³⁵S]Met-tRNA_i associated with the ribosome. We also found that α - γ dimers were indistinguishable from the complete trimer with regard to their capacity to stimulate [³⁵S]Met-tRNA_i binding to ribosomes which agreed with their Met-tRNA_i binding competence. Interestingly, the isolated γ -subunit was also able to stimulate the interaction of [³⁵S]Met-tRNA_i with the small ribosomal subunit, although to a lesser extent than the α - γ dimer or the complete trimer. This latter result indicated that the γ -polypeptide has some intrinsic capacity for

interacting with Met-tRNA_i as well as with the ribosome independently of the presence of the other two subunits.

GTP hydrolysis

Lastly, we examined the requirements for triggering hydrolysis of GTP bound to the $\alpha/\text{eIF2}$ trimer. First, $\alpha/\text{eIF2}$ was pre-incubated with GTP and Met-tRNA_i at 65°C to allow formation of the ternary complex. Then, we added to the mixture 70S ribosomes and an oligo-ribonucleotide comprising a strong translation initiation site. In parallel, we verified by non-denaturing gel electrophoresis the ability of the oligo-ribonucleotide to bind to ribosomes (data not shown). The reaction was continued and samples were withdrawn at different times upon incubation. Under these conditions, no significant GTP hydrolysis was observed (data not shown), suggesting that the interaction of the factor with the ribosome, even in the presence of codon-anticodon pairing, is not sufficient by itself to trigger the reaction. This situation is reminiscent to eukaryotes, where GTP hydrolysis on eIF2 requires the auxiliary protein eIF5. However, an eIF5 homologue is apparently lacking in archaea, raising the possibility that a hitherto unidentified factor is involved in hydrolysis of GTP bound to $\alpha/\text{eIF2}$.

DISCUSSION

In this study, we have analysed several functional aspects of the eIF2 homologue from the thermophilic archaeon *S.solfataricus*. The purified recombinant $\alpha/\text{eIF2}$ subunits readily assembled *in vitro* to form dimeric and trimeric complexes. As observed previously for *P.abyssii* $\alpha/\text{eIF2}$ (18), we found that the *S.solfataricus* $\alpha/\text{eIF2}$ γ -subunit constitutes the structural core of the trimeric complex since it interacts with both α - and β -subunits as well as individually with either of them.

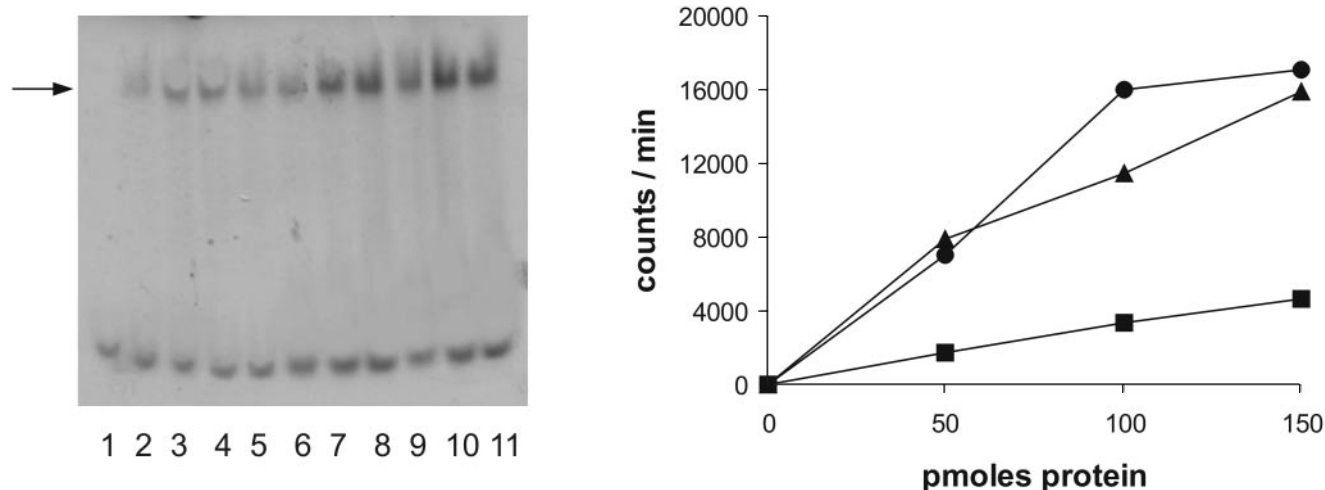


Figure 7. Stimulation of M-tRNA_i binding to ribosomes by $\alpha/\text{eIF2}$: 30 pmol of 70S ribosomes were incubated with [³⁵S]Met-tRNA_i and increasing amounts of $\alpha/\text{eIF2}$ (complete trimer, α - γ dimer or the γ -subunit only). The samples were analysed on a non-denaturing polyacrylamide gel (left panel). Lane 1, [³⁵S]Met-tRNA_i alone; lane 2, 70S ribosomes were incubated with [³⁵S]Met-tRNA_i without $\alpha/\text{eIF2}$; lanes 3–5, 70S ribosomes were incubated with [³⁵S]Met-tRNA_i in the presence of 50 (lane 3), 100 (lane 4) and 150 (lane 5) pmol of the γ -subunit; lanes 6–8, 70S ribosomes were incubated with [³⁵S]Met-tRNA_i in the presence of 50 (lane 6), 100 (lane 7) and 150 (lane 8) pmol of the α - γ dimer; and lanes 9–11, 70S ribosomes were incubated with [³⁵S]Met-tRNA_i in the presence of 50 (lane 9), 100 (lane 10) and 150 (lane 11) pmol of the trimer. The position of the 30S ribosomal subunit (arrow) was determined separately by Coomassie blue staining. The amount of tRNA bound to ribosomes was quantified using an Instant Imager apparatus (right panel). The total amount of radioactivity in each lane is somewhat variable owing to deacylation undergone by free Met-tRNA_i during the run. Circles, complete trimer; triangles, α - γ dimer; and squares, γ -subunit.

In contrast, the α - and β -subunits are unable to interact with each other independent of the γ -polypeptide (Figure 2). Protein-protein recognition is independent of both GTP and high temperature. The recombinant trimeric *S.solfataricus* a/eIF2 displays properties very similar to those of its eukaryal counterpart: it interacts with Met-tRNA_i (Figure 5), and stimulates its binding to the small ribosomal subunits (Figure 7). Taken together with the results of Yatime *et al.* (18), we therefore conclude that a/eIF2 is the principal tRNA_i binding factor in archaea.

The archaeal and eukaryal IF2 factors display several notable functional differences, which may aid towards a better understanding of the evolutionary history of this protein. One difference concerns the tRNA_i-binding site. According to a recent crystallographic analysis, the γ -subunit of *P.abysssi* a/eIF2 includes an apparently complete tRNA_i-binding domain similar to that of elongation factor Tu, another translational G-protein with tRNA-binding properties (19,22). In agreement with this finding, the γ -subunit of a/eIF2 does possess some intrinsic capacity for Met-tRNA_i binding: it protects the tRNA-bound methionine from alkaline hydrolysis (18), and it can promote to some extent binding of Met-tRNA_i to the 30S ribosomal subunits (Figure 7). Nevertheless, a stable interaction with Met-tRNA_i is only achieved by a α - γ dimer, and therefore requires the presence of the α -subunit (Figure 5A) (18). It has been found that the C-terminal portion of the α -subunit is sufficient for stable tRNA binding (18). However, the precise role of the α -subunit in Met-tRNA_i binding by the trimeric a/eIF2, or by the α - γ complex, remains to be elucidated. In contrast, in eukaryal IF2 the Met-tRNA_i binding site seems to be shared between the γ - and the β -subunits (23,24), while the main function of the α -subunit is the regulation of the factor activity (25). It should be pointed out that the eukaryal β -subunit is about twice the size of its archaeal counterpart, and, besides Met-tRNA_i binding, is implicated in several other essential tasks, such as controlling the interaction with eIF5 and eIF2B (14–16), and possibly mRNA binding (26). Only the C-terminal half of the eukaryal polypeptide is conserved in the archaeal one, while the N-terminal moiety, containing the interaction domains for eIF2B and eIF5, has been apparently added during eukaryotic evolution. Therefore, if the eukaryal β -subunit is involved in the interaction with tRNA_i, this function is most likely acquired during eukaryotic evolution and not present in the ancestral β -subunit of a/eIF2.

Another relevant functional difference between a/eIF2 and eIF2 concerns the binding of guanine nucleotides. The eukaryal protein has a much higher affinity for GDP than for GTP and requires a GDP/GTP exchange factor, the pentameric protein eIF5B, to be reactivated after GTP hydrolysis. In contrast, the analysis presented here of guanine nucleotide binding and exchange on a/eIF2 allows the conclusion that the archaeal factor has a similar affinity for GDP and GTP (Figures 3 and 4), and therefore might not require an exchange factor. This agrees with the observation that archaeal genomes lack a complete eIF5B homologue (21). It is also worth noting that in lower eukaryotes (yeast), eIF2 seems to have a reduced requirement for the exchange factor when compared with the mammalian protein (27). These data suggest the possibility that the free-nucleotide-exchanging archaeal a/eIF2 represents the prototype factor from which an exchange factor-dependent one has evolved in eukaryotes, which provided additional

or even a more sophisticated translational control. Taken together, these observations would suggest that no eukaryal-type functional regulation of a/eIF2 based on the inhibition of guanine nucleotide exchange exists in archaea. However, it has been recently reported that the α -subunit of *Pyrococcus horikoshii* a/eIF2 is phosphorylated by a specific protein kinase (28). Thus, the determination of the function of α -subunit phosphorylation of archaeal a/eIF2 will be an interesting task, which may help to clarify why archaea, unlike bacteria but like eukarya, have adopted a trimeric Met-tRNA_i binding factor.

Another unresolved problem concerns GTP hydrolysis. Given the lack of an eIF5 homologue, our expectation was to find a ribosome-triggered, but otherwise spontaneous, hydrolysis of a/eIF2-bound GTP. However, all our attempts to reveal *in vitro* the presence of such a mechanism failed. Therefore, the possibility exists that archaea possess a domain-specific auxiliary GTPase, different from eIF5. Some years ago, Kyrpides and Woese (21) suggested that the auxiliary GTPase of a/eIF2 might be aIF2, the universal factor homologous to bacterial IF2 and eukaryal eIF5B. The aIF2 protein is a G protein and does possess a ribosome-dependent GTPase activity (A. Berardi, S. Marzi, E. Maone, L. Peri, C. O. Gualerzi, P. Cammarano and P. Londei, unpublished data).

An interesting outcome of our study is the general conclusion that most of the functions of the factor are carried out by its largest component, the γ -subunit. At least *in vitro*, the γ -subunit has retained some intrinsic ability to interact with Met-tRNA_i, to bind to the small ribosomal subunits, and to promote the interaction between Met-tRNA_i and the ribosome. The α -subunit is clearly involved in stabilizing Met-tRNA_i binding, while the function of the small β -subunit remains elusive at the moment. On the whole, these observations suggest that the γ -subunit functioned as a monomer early in evolution, preceding the time when the archaeal–eukaryal line split into two separate domains. This hypothesis agrees with the fact that the γ -subunit of eIF2-like proteins belongs to a family of tRNA-binding G-proteins that includes the elongation factor Tu/1-A and its specialized version SELB (29). The latter is the factor that selects selenocysteine tRNA during the synthesis of selenoproteins in all primary domains (30–32). The γ -subunit of eukaryal and archaeal IF2 is most closely related to SELB (29), suggesting that the a/eIF2 γ -subunit arose by gene duplication from elongation factor Tu at about the same time as SELB. Each of the two proteins became then specialized for interacting with a single aminoacyl-tRNA species, whereas the elongation factor Tu retained the capacity to bind any aminoacyl-tRNA. Probably, a/eIF2 acquired a selective affinity for Met-tRNA_i during the evolution of the common ancestor of Archaea and Eukarya. However, a/eIF2 must soon have acquired its present trimeric form, as genes encoding the α - and β -subunits are found in all archaea, including the hyperthermophile *Nanoarchaeum equitans*, which has a genome size of only 0.5 Mb (33).

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Conflict of interest statement. None declared.

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