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Functional Molecular Mapping of Archaeal Translation Initiation Factor 2*

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Eukaryotic and archaeal initiation factors 2 (e/aIF2) are heterotrimeric proteins ($\alpha\beta\gamma$) carrying methionylated initiator tRNA to the small subunit of the ribosome. The three-dimensional structure of aIF2γ from the Archaea *Pyrococcus abyssi* was previously solved. This subunit forms the core of the heterotrimer. The α and β subunits bind the γ, but do not interact together. aIF2γ shows a high resemblance with elongation factor EF1-A. In this study, we characterize the role of each subunit in the binding of the methionylated initiator tRNA. Studying various aminoacyl-tRNA ligands shows that the methionyl group is a major determinant for recognition by aIF2. aIF2γ alone is able to specifically bind Met-tRNA$_i^{Met}$, although with a reduced affinity as compared with the intact trimer. Site-directed mutagenesis confirms a binding mode of the tRNA molecule similar to that observed with the elongation factor. Under our assay conditions, aIF2β is not involved in the docking of the tRNA molecule. In contrast, aIF2α provides the heterotrimer its full tRNA binding affinity. Furthermore, the isolated C-domain of aIF2α is responsible for binding of the α subunit to γ. This binding involves an idiosyncratic loop of domain 2 of aIF2γ. Association of the C-domain of aIF2α to aIF2γ is enough to retrieve the binding affinity of tRNA for aIF2. The N-terminal and central domains of aIF2α do not interfere with tRNA binding. However, the N-domain of aIF2α interacts with RNA unspecifically. Based on this property, a possible contribution of aIF2α to formation of a productive complex between aIF2 and the small ribosomal subunit is envisaged.

In the initiation of translation, the recognition of a start codon on a mRNA involves a specialized initiator tRNA. This tRNA is always esterified with methionine. Several proteins, called initiation factors, contribute along with the ribosome to the success of the initiator tRNA-mRNA interaction. Such initiation factors differ in the three domains of life.

The case of bacteria is apparently the simplest, with three initiation factors involved, IF1,[1] IF2, and IF3. Among these, IF2 ensures the carrying of f-Met-tRNA$_f^{Met}$ toward the P site on the ribosome (1).

In Eukarya, translation begins at the capped 5′ end of mRNA with a 43 S ribosomal complex containing the small 40 S ribosomal subunit, an eIF2-GTP-Met-tRNA$_i^{Met}$ complex and four initiation factors (eIF4A, eIF4B, eIF4F, and eIF3) (2–5). Further recruitment of eIF1 and eIF1A enables this complex to scan the mRNA until an AUG codon is encountered (6, 7). In the resulting 48 S complex, eIF5 interacts with eIF2β and induces hydrolysis of the GTP molecule bound to eIF2 (8–10). As a result, eIF2-GDP is released and the 60 S large ribosomal subunit binds to the 40 S subunit to produce the competent elongator 80 S ribosome in the presence of eIF5B (11–14). Intriguingly, eIF5B closely resembles bacterial IF2 (15).

The mechanism of initiation of translation in Archaea is still poorly known. However, several features can be deduced from genomic comparisons (16, 17). Characteristics from both the bacterial and the eukaryal domains are encountered. As in bacteria, the small ribosomal subunit is prepositioned in the vicinity of the AUG start codon through pairing of 16 S rRNA with a Shine-Dalgarno sequence. In contrast to bacteria, but as in Eukarya, the methionine esterified to initiator tRNA is not formylated. Possibly in line with this property, the heterotrimeric aIF2 protein, responsible for transport of initiator tRNA, resembles eukaryal eIF2, not a bacterial IF2 (18, 19). As in Eukarya, eIF5B, a homologue of eubacterial IF2, exists in Archaea (20). Orthologs of eIF1, eIF1A, eIF5A, and eIF6 are clearly present. Orthologs of eIF4, eIF5, eIF2B, and eIF3 are not found (18, 19).

e/aIF2 results from the association of three subunits, α, β, and γ. The γ subunit forms the core of the heterotrimer. It interacts with both the α and β subunits. In the trimer, the α and β subunits do not interact with one another (21). The three-dimensional structure of the γ subunit was recently determined in the case of the archaeon *Pyrococcus abyssi*. This subunit displays a GTP binding site and strongly resembles active EF-Tu (21) (Fig. 1). This homology led to the proposal that tRNA is recognized by aIF2γ in a manner similar to that encountered in EF-Tu.

aIF2β is made of two domains. The structures of these domains are known in the case of *Methanococcus jannaschii* aIF2β. A zinc finger in the C-terminal domain is involved in the selection of the start codon, probably through mRNA binding (22, 23). The N-terminal domain participates in the binding of the γ subunit (10). A possible role of eIF2β in the binding of tRNA remains controversial (24, 25). Notably, the polylysine motifs found at the N terminus side of eIF2β are absent in archaeal aIF2β. In eucarya, these motifs are essential to the association with eIF5 (8–10).

e/aIF2α is made of three domains. The structure of the N-terminal segment of human eIF2α was recently determined (26). This segment comprises two of the eIF2α domains, an N-terminal β-barrel followed by a helical domain. The structure of the third domain is not known. In the β-barrel, a mobile

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[1] The abbreviations used are: IF, initiation factor; eIF, eukaryotic initiation factor; e/aIF, eukaryotic and archaeal initiation factors; Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; MOPS, 4-morpholinepropanesulfonic acid.

FIG. 1. **Three-dimensional structure of aIF2γ complexed with Gpp(NH)p-Mg^{2+} (21).** The three domains are labeled. In the G-domain, the Gpp(NH)p molecule, a Mg^{2+} ion, and a zinc ion are represented. The *two loops* of domain 2 that distinguish the initiation factor from elongation factor EF1-A are in *dark*. The three β-strands, which we propose to form the methionine binding pocket, are also represented in *dark*. The figure was drawn with Setor (54).

loop harbors a serine that is the target of specific kinases in response to several conditions of stress (reviewed in Refs. 27–30). This serine is conserved in all eucaryal eIF2α sequences, not in the archaeal aIF2α ones. In eucarya, the eIF2α subunit is thought to play an important role in the control of the action of the nucleotide exchange factor eIF2B (31). In Archaea, eIF2B has no equivalent, and nucleotide exchange on aIF2 is thought to occur spontaneously. Notably, in *Saccharomyces cerevisiae*, mutations in the *SUI2* gene encoding eIF2α were found to cause a relaxed start codon specificity in the corresponding strain. Indeed, in the mutant strain, translation of *HIS4* can be initiated at a UUG codon (32).

In this study, to evaluate the role of each aIF2 subunit in tRNA selection, the affinities of aminoacylated tRNA for aIF2γ alone or in combination with either aIF2α or aIF2β, were measured. aIF2γ binds methionylated initiator tRNA with tight recognition of the methionyl moiety in a pocket of domain 2. Under our assay conditions, aIF2β does not participate in tRNA binding. On the other hand, the addition of aIF2α strongly reinforces the affinity of aIF2γ for methionylated initiator tRNA. We show that the C-terminal domain of aIF2α associates with a loop of aIF2γ. The addition of this C-domain to aIF2γ is enough to retrieve full tRNA binding affinity. Although it does not contribute to tRNA binding, the isolated N-terminal domain of aIF2α shows general RNA binding properties. This property suggests a role of aIF2α in rRNA or mRNA binding, which might be related to the known importance of this subunit for the selection of the start codon (32).

EXPERIMENTAL PROCEDURES

Expression and Purification of aIF2 Subunits—Wild-type α, β, and γ subunits of aIF2 were expressed in *Escherichia coli* BL21 cells from pET3a-based vectors. The proteins were purified as described (21). Stoichiometric amounts of each subunit were mixed to obtain an αγ, βγ dimer, or αβγ trimer. Stoichiometry and proper assembly of the oligomers were verified by PAGE analysis under native and denaturing conditions. Mutants of aIF2α and aIF2γ were constructed by using PCR-based site-directed mutagenesis of the cloned genes (Stratagene, La Jolla, CA). To construct the γΔ*L1* gene, the sequence corresponding to residues 221–227 of aIF2γ was replaced by a glycine codon. To construct the γΔ*L2* gene, the sequence corresponding to residues 256–265 of aIF2γ was replaced by a glycine codon. To obtain αD1, the codon corresponding to Gln^{88} in the α subunit was replaced by a TGA stop codon. The DNA encoding αD1–2 was obtained by replacing the codon corresponding to Val-173 with a TGA stop codon. The DNAs coding for αD2–3 and αD3 were obtained by PCR amplification with the introduction of a start codon in position 88 or a start codon in position 173, respectively, followed by recloning of the amplification product into a pET3a derivative.

TABLE I
Dissociation constants of aminoacylated tRNAs from their complexes with wild-type aIF2

The values are derived from the plot of the deacylation rates as a function of the concentration of aIF2 measured at 51 °C in the presence of a fixed aminoacyl-tRNA concentration (80 nM) and of 1 mM Gpp(NH)p (see "Experimental Procedures"). tRNA f^{Met} is the *E. coli* initiator tRNA.

	tRNA variants	K_d
		nM
1	Met-tRNA i^{Met} *P. abyssi*	90 ± 30
2	Met-tRNA f^{Met}	120 ± 40
3	Met-tRNA f^{Met} A_1-U_{72}	90 ± 30
4	Met-tRNA m^{Met}	450 ± 100
5	Met-tRNA 1^{Val} CAU	400 ± 100
6	Val-tRNA 1^{Val}	> 50,000
7	Val-tRNA f^{Met} UAC	> 50,000
8	Met-tRNA f^{Met} G_1-C_{72}	320 ± 80

Mutant aIF2γ proteins were expressed and purified as described above for the wild-type subunit. Mutants of truncated aIF2α proteins corresponding to αD1 and αD3 were purified as described for the full-length α subunit (21). In the cases of αD1–2 and αD2–3, the Mono S step was replaced by a Mono Q step, according to the acidic isoelectric point of these proteins (6.5 for both). In all cases, mutant proteins were overexpressed in soluble, thermostable form and at high levels (final yield of about 20 mg of protein per liter of culture). This indicates that all mutants were stably folded.

Protection Assay—$tRNA_f^{Met}$, $tRNA_i^{Val}$, and their variants were produced in *E. coli* from constructed genes, as described (33, 34). The gene for $tRNA_i^{Met}$ from *P. abyssi* was constructed by assembly of oligonucleotides and inserted into pBSTNAV (35). tRNAs were purified by anion exchange chromatography (35). Amino acid acceptor capacities were between 800 and 1400 pmol per A_{260} unit. Full aminoacylation with [^{35}S]methionine (10,000 dpm/pmol, PerkinElmer Life Sciences) was achieved using homogeneous *E. coli* Met^{547} MetRS (36). Valylation with [^{3}H]Val (4500 dpm/pmol, Amersham Biosciences) was performed with homogeneous *E. coli* ValRS (37). 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 were systematically determined after precipitation in 5% trichloroacetic acid.

Protection by aIF2 of aminoacylated tRNAs against spontaneous hydrolysis was performed as follows. Reaction mixtures (150 μl) contained 20 mM Hepes-NaOH, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.2 mg/ml bovine serum albumin (Roche Applied Sciences), 5% glycerol, 1 mM Gpp(NH)p and 80 nM of the aminoacylated tRNA under consideration. Concentrations of aIF2 or of its variants were varied from 20 nM to 50 μM according to the K_d value to be measured. The mixtures were incubated at 51 °C. To determine the rate constant of deacylation, 25-μl aliquots were withdrawn at various times (5 to 30 min) and precipitated in 5% trichloroacetic acid in the presence of 80 μg of yeast RNA as carrier. In all cases, the deacylation curve as a function of time could be fitted with a single exponential. Rate constants measured at variable protein concentrations were fitted to simple binding curves from which the dissociation constant of the studied protein-tRNA complexes could be deduced using the MC-Fit program (38). Typical experiments are illustrated in Fig. 3. The concentrations of aIF2γ or of heterotrimeric aIF2 were determined by measuring the zinc content in the protein preparations by atomic absorption spectroscopy as described (21). Indeed, aIF2γ contains one zinc ion, whereas aIF2 contains two zinc ions. The percentage of active protein was then measured by making a protection test of 1 μM $Met\text{-}tRNA_f^{Met}$ in the presence of 0.8 μM aIF2. From the determination of the amount of protected tRNA, it was concluded that aIF2 was 100% active. This indicates in addition that our aIF2 preparations do not contain residual GDP.

Native Gel Electrophoresis and Gel Shift Assay—aIF2 α, β, and γ subunits display basic isoelectric points of 8.7, 8.0, and 8.0, respectively. The isoelectric point of αD1 and αD3 is 9.6, that of αD1–2 and αD2–3 is 6.5. To follow molecular assembly between subunits or their variants, acidic native gels were prepared. Each gel contained a separation part with 12% acrylamide (37.5:1, Bio-Rad) at pH 4.3 (120 mM KOH plus 72 mM acetic acid) and a stacking region composed of 4% acrylamide at pH

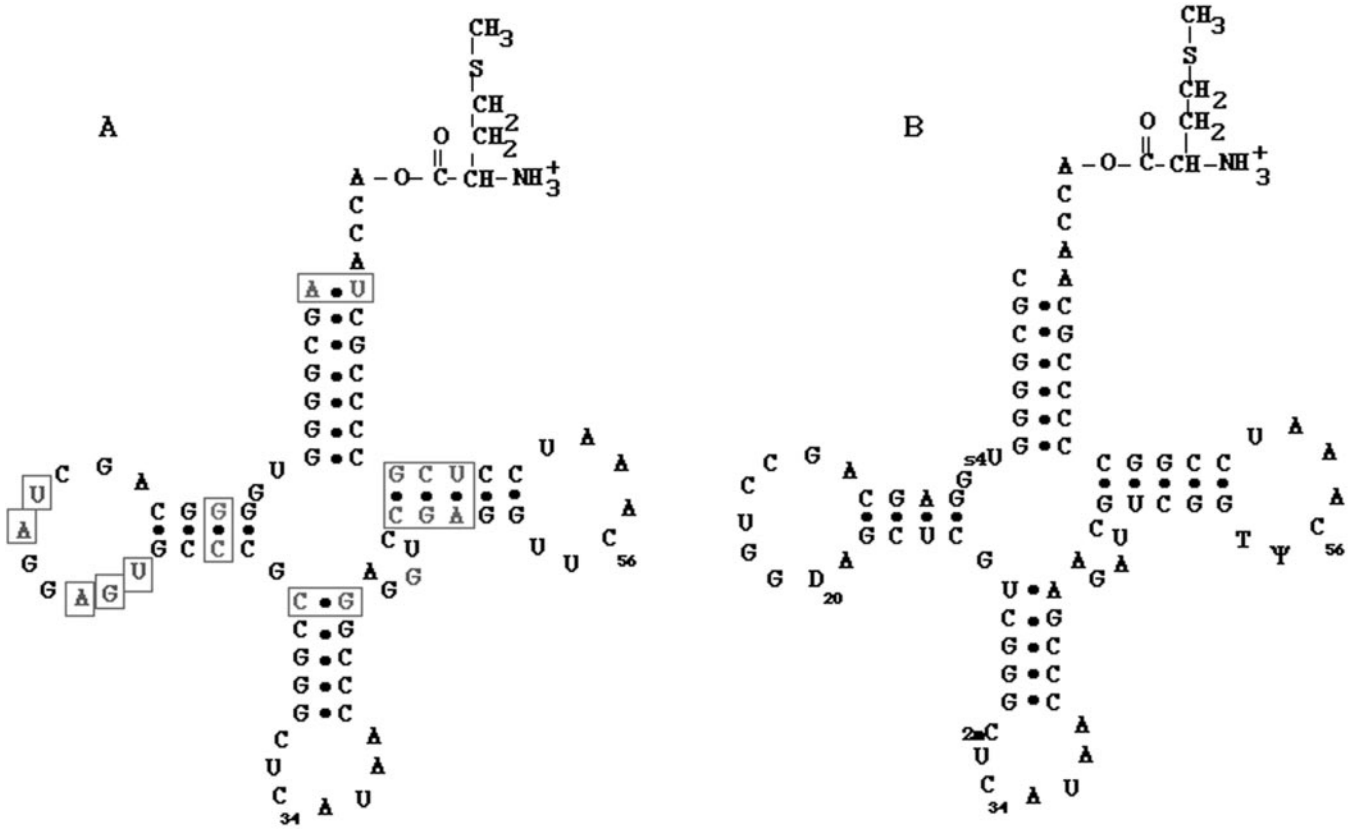

FIG. 2. **Cloverleaf representations of *P. abyssi* $tRNA_i^{Met}$ (*A*) and *E. coli* initiator $tRNA_f^{Met}$ (*B*).** Nucleotides that differ in *P. abyssi* $tRNA_i^{Met}$ and *E. coli* initiator $tRNA_f^{Met}$ are *boxed* and typed in *gray*. Post-transcriptional modifications of *P. abyssi* $tRNA_i^{Met}$ are not known (55).

TABLE II
Dissociation constants of Met-tRNA f^{Met} or Val-tRNA f^{Met} UAC (80 nM) from their complexes with the indicated combinations of wild-type (wt) or mutant aIF2 subunits

The K_d values are derived from the plot of the deacylation rates as a function of variable concentrations of the studied proteins as measured at 51°C in the presence of 1 mM Gnp(NH)p (see "Experimental Procedures"). A dash indicates that the corresponding subunit is absent.

	α	β	γ	*Kd*
				nM
1	—	—	wt	5,000 ± 3,000[a]
2	—	—	wt	>80,000[b]
3	—	wt	wt	5,000 ± 3,000[a]
4	wt	—	wt	44 ± 20[a]
5	wt	wt	wt	120 ± 40[a]
6	—	—	L1	>50,000[a]
7	wt	—	L1	>50,000[a]
8	wt	wt	L2	130 ± 60[a]
9	wt	wt	G235D	>50,000[a]
10	D1	—	wt	10,000 ± 4,000[a]
11	D1·2	—	wt	10,000 ± 4,000[a]
12	D3	—	wt	42 ± 20[a]

[a] Met-tRNA f^{Met}.
[b] Val-tRNA f^{Met} UAC.

6.8 (120 mM KOH, 12 mM acetic acid). The gel was run in a buffer, pH 4.4, containing 133 mM acetic acid and 350 mM β-alanine during 1 h at 150 V at room temperature in a Protean II system (Bio-Rad). Proteins were revealed with Coomassie Brillant Blue. The observed migrations of the isolated subunits, of the dimers and trimer, were consistent with their molecular ratios.

Gel shift assays were performed by mixing various amounts of the studied protein with *E. coli* $tRNA_f^{Met}$ (4 μM final concentration) in 50 mM TBE buffer, pH 8.3, containing 200 mM NaCl, 1 mM $MgCl_2$, and 10 mM β-mercaptoethanol. The volume of the applied samples was 20 μl. For competition experiments, various amounts of *E. coli* 16 S + 23 S rRNA (Roche Applied Sciences) were added to $tRNA_f^{Met}$ and protein (see legend of Fig. 4).

Molecular Sieving—Purified wild-type or mutant subunits (in the 10 μM range) were mixed pairwise or all three together in 10 mM MOPS, pH 6.7, 10 mM 2-mercaptoethanol, and 500 mM NaCl. Each individual protein or mixture of proteins (αβ, βγ, αγ, αβγ, βγΔL1, αγΔL1, αβγΔL1, βγΔL2, αγΔL2, αD1γ, αD1–2γ, αD3γ) were chromatographed onto a TSK3000 SWXL column equilibrated in the same buffer (Tosohaas, Japan, 7.8 × 3000 mm^2). Elution was performed at 0.7 ml/min. An example is shown in Fig. 6.

Circular Dichroism—Circular dichroism spectra of aIF2γ proteins at a concentration of 2.5 μM in 10 mM potassium phosphate, pH 6.7, plus 100 mM NaCl were recorded at 20 °C in a Jasco J710 spectropolarimeter (2 nm bandwidth, 1-mm path cuvette).

RESULTS

Binding of Initiator Met-tRNA to aIF2—Protection of aminoacylated tRNA by EF-Tu against spontaneous deacylation is well documented (39). Consequently, one expects that aIF2 can also protect archaeal Met-tRNA. To evidence such an effect, we overexpressed in *E. coli* a gene corresponding to the initiator tRNA of *P. abyssi*. The product of this gene, $tRNA_i^{Met}$, was purified and assayed for its ability to bind heterotrimeric aIF2 after aminoacylation with [^{35}S]methionine. At 51 °C, Met-$tRNA_i^{Met}$ (80 nM) deacylated spontaneously at a rate of 0.28 min^{-1}. In the presence of aIF2 (5 μM) and of the GTP analogue Gpp(NH)p (1 mM), strong protection was observed. The measured deacylation rate was reduced to a value lower than 0.009 min^{-1}. Upon Gpp(NH)p omission, the protection was lost. With GDP instead of Gpp(NH)p, the deacylation rate was 0.11 min^{-1} (5 μM aIF2 and 1 mM GDP-Mg^{2+}). By measuring deacylation rates in the presence of various concentrations of aIF2, a dissociation constant of Met-$tRNA_i^{Met}$ from the aIF2-Gpp(NH)p-Met-$tRNA_i^{Met}$ complex could be deduced. The K_d value is 90 ± 30 nM (Table I, row 1). Notably, this K_d value is identical when GTP is used instead of the non-hydrolyzable Gpp(NH)p analogue. This indicates that under the assay conditions, no significant spontaneous GTP hydrolysis occurs, and suggests that efficient GTP hydrolysis by aIF2 is related to start codon se-

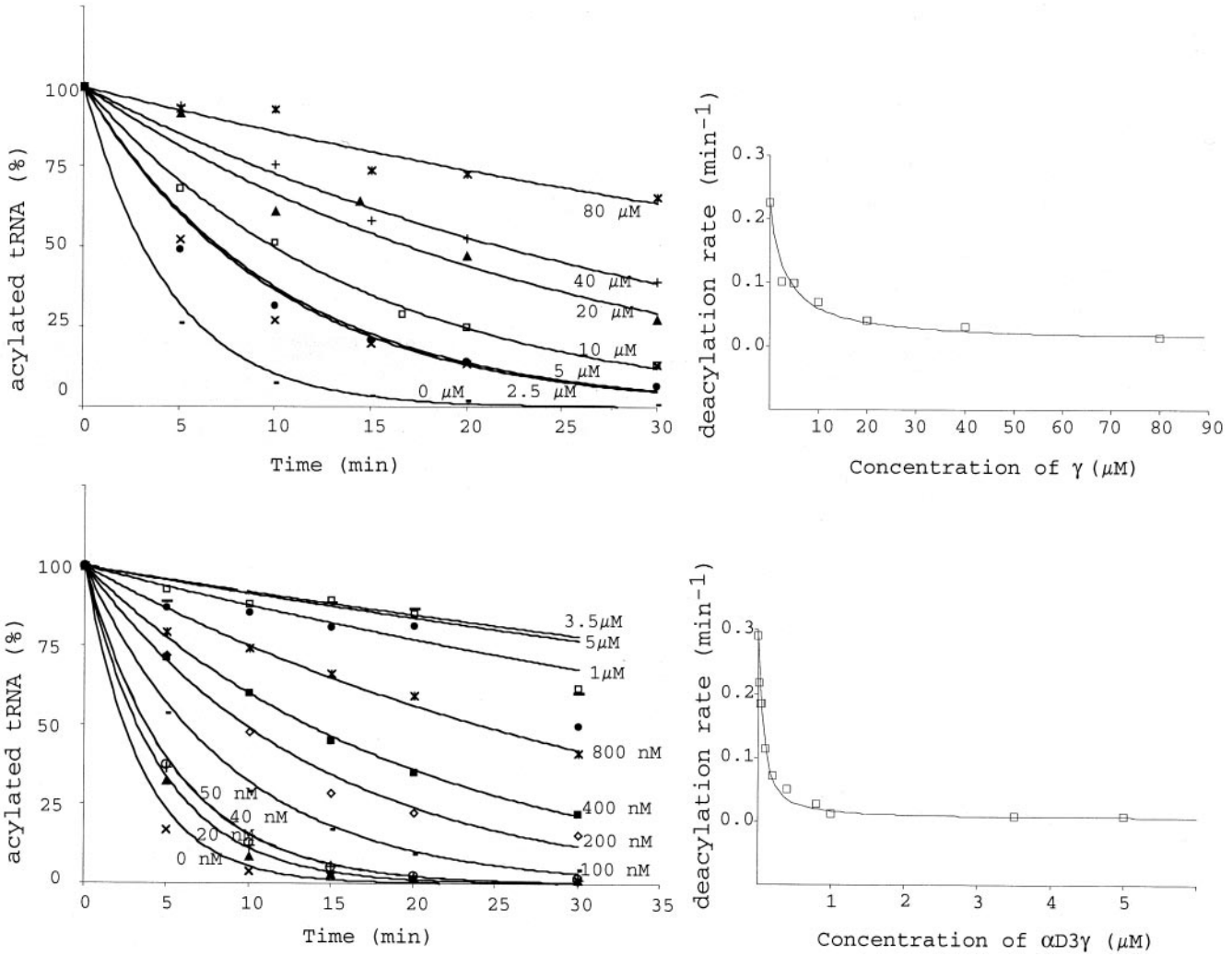

FIG. 3. **Determination of the dissociation constant (K_d) of Met-tRNA$_f^{Met}$ from its complexes with aIF2γ (*top*) or with an aIF2αD3γ dimer (*bottom*).** The *left panels* show the kinetics of tRNA deacylation measured in the presence of a variable protein concentration. Each deacylation curve was fitted to a single exponential from which one deacylation rate was derived. The *right panels* show the plots of the derived rate constants as a function of protein concentration. Each set of data were fitted to a binding curve from which a dissociation constant of tRNA from its complex was derived (see "Experimental Procedures" as well as Tables I and II).

lection on the ribosome, as this is the case for eukaryal eIF2 (12).

As shown in Fig. 2, initiator tRNA from *P. abyssi* closely resembles that from *E. coli*. The sequences of the acceptor stems are identical, the only exception being a weak A^1-U^{72} base pair in the archaeal tRNA instead of the unpaired C^1-A^{72} bases of bacterial tRNAs. In agreement with this resemblance, when added in the protection assay, *E. coli* Met-tRNA$_f^{Met}$ appeared as good a ligand of *P. abyssi* aIF2 as the *P. abyssi* initiator tRNA produced in *E. coli* (Table I, rows 1 and 2). Moreover, the change of the C^1-A^{72} mismatch of *E. coli* tRNA$_f^{Met}$ into a A^1-U^{72} base pair, as in an archaeal tRNA, had no significant effect on the binding affinity to aIF2 (Table I, rows 2 and 3). We concluded that the highly conserved A^1-U^{72} base pair of archaeal and eucaryal initiator tRNAs is not required for aIF2 binding. Finally, this comparison validates the use of *E. coli* Met-tRNA$_f^{Met}$ as a model ligand to study complex formation between aIF2 and tRNA.

Specificity of the assay based on deacylation of an aminoacylated tRNA was evaluated using several aminoacyl-tRNAs. First, protection against deacylation of *E. coli* Val-tRNA$_1^{Val}$ could not be observed, even in the presence of 50 μM aIF2 (Table I, row 6). Specificity of the factor toward the methionine moiety of the initiator Met-tRNA was established by using a tRNA$_f^{Met}$ variant with a UAC anticodon instead of the CAU one. This variant can be aminoacylated with valine in the presence of *E. coli* ValRS (33, 40). The resulting Val-tRNA$_f^{Met}$ UAC escapes protection against deacylation, even in the presence of up to 50 μM aIF2 (Table I, row 7). Therefore, with valine instead of methionine, the binding affinity of aminoacylated initiator tRNA appears to have been reduced by at least 2 orders of magnitude.

Two other tRNAs aminoacylated with methionine, distinct from initiator tRNA, were also assayed for their resistance to deacylation in the presence of aIF2. The first one was *E. coli* elongator Met-tRNA$_m^{Met}$. The second one was a derivative of tRNA$_1^{Val}$ carrying a CAU anticodon that allows its aminoacylation by MetRS (40). Although different from initiator tRNA in their nucleotide sequences, these two methionylated tRNAs are efficiently protected against deacylation (Table I, rows 4 and 5). We therefore concluded that the methionyl moiety plays a crucial role in the binding to the factor. However, the dissociation constants of the two above methionylated tRNAs are increased by a factor of 4, as compared with Met-tRNA$_f^{Met}$. This difference indicates some participation of the polynucleotide itself to aIF2 binding. To test this idea, an *E. coli* tRNA$_f^{Met}$ variant with a G^1-C^{72} base pair instead of the wild-type unpaired C^1-A^{72} bases was prepared. aIF2 protects this tRNA

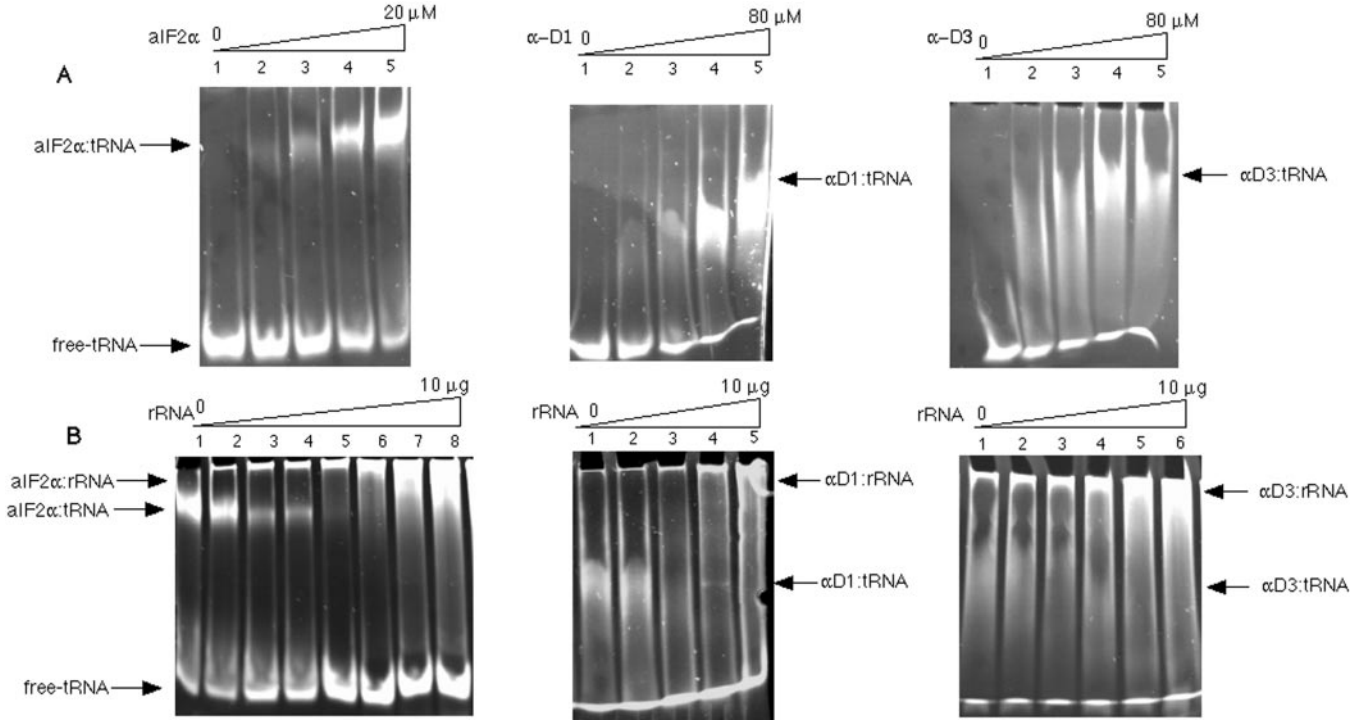

FIG. 4. **Gel shift analysis of the RNA binding properties of the three domains in aIF2α.** *Panel A*, binding to $tRNA_f^{Met}$: *left*, 1.8 μM tRNA was mixed in 20 μl of 200 mM NaCl, 50 mM TBE, pH 8.3, 1 mM $MgCl_2$, 500 μM Gpp(NH)p, and 10 mM β-mercaptoethanol, with increasing concentrations of aIF2α (0, 2.5, 5, 10, and 20 μM; *lanes 1–5*). *Middle*, the same experiment was performed with αD1, except that protein concentrations were 0, 10, 20, 40, and 80 μM (*lanes 1–5*). *Right*, same experiment as in the *middle panel w*ith αD3 instead of αD1. After a 15-min incubation at room temperature, samples were loaded onto a 5% polyacrylamide gel containing 50 mM TBE and 1 mM $MgCl_2$. Electrophoresis was in the same buffer. The gels were stained with ethidium bromide. *Panel B*, competition of rRNA with tRNA binding to aIF2α domains. *Left*, aIF2α (20 μM, 13.6 μg) was mixed with 1 μg (1.8 μM) of $tRNA_f^{Met}$ in 20 μl of 200 mM NaCl, 50 mM TBE, pH 8.3, 1 mM $MgCl_2$, 500 μM Gpp(NH)p, and 10 mM β-mercaptoethanol. *Lanes 1–8* show the results with increasing amounts (none, 0.2, 0.5, 1, 2, 4, 7.5, and 10 μg, respectively) of 16 S + 23 S *E. coli* rRNA. *Middle*, same experiment but with αD1 (80 μM) instead of aIF2α. Amounts of rRNA were 1, 2, 4, 7.5, and 10 μg (*lanes 1–5*, respectively). *Right*, same experiment with αD3 (80 μM). Amounts of rRNA were 0.5, 1, 2, 3, 5, and 7.5 μg (*lanes 1–6*, respectively). The gels were stained with ethidium bromide. Note that Coomassie Blue staining of the same gels always revealed the presence of protein in the shifted tRNA bands as well as in the rRNA bands. aIF2α, αD1, and αD3 proteins alone do not migrate in the gels, in agreement with their basic isoelectric points (see "Experimental Procedures"). Notably, under the same gel conditions, aIF2β does not promote any observable RNA shift.

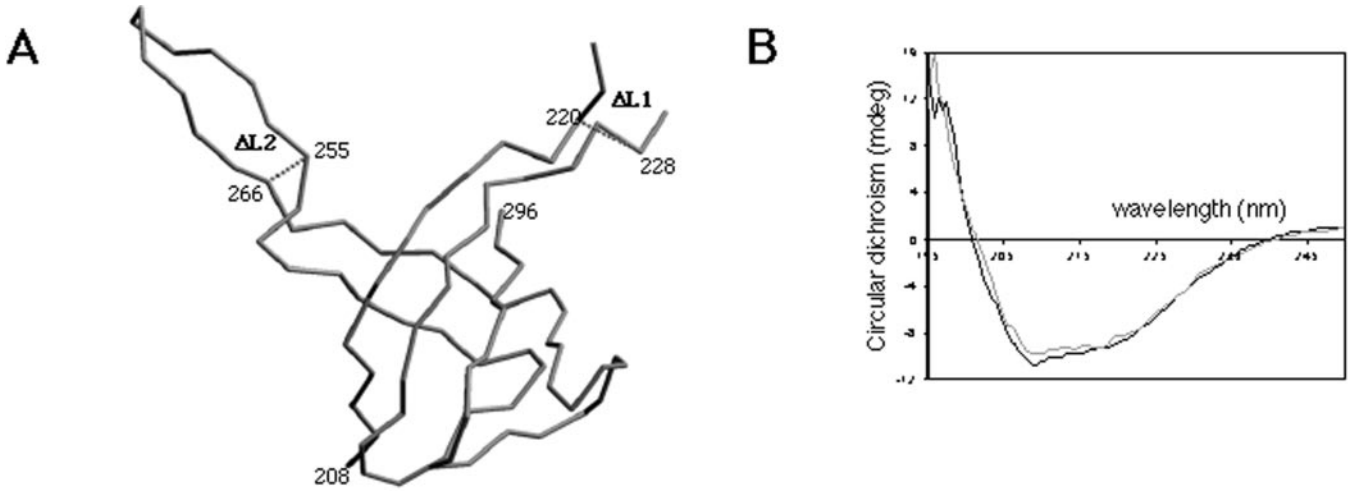

FIG. 5. *Panel A*, locations of ΔL1 and ΔL2 deletions on the structure of domain 2 of aIF2γ. The Cα trace of domain 2 of aIF2γ is drawn (residues 208–296 (21)). The two loops protrude outside of the barrel and are not involved in interactions with the core of the barrel. Note that in the crystals, loop L1 was disordered between residues 223 and 229, showing its mobility. Mutation ΔL1 corresponds to the replacement of residues 221–227 by a glycine. The distance between the Cα atoms of residues 220 and 228 in the structure is 7.6 Å. Mutation ΔL2 corresponds to the replacement of residues 256–265 by a glycine. The distance between the Cα atoms of residues 255 and 266 in the structure is 5 Å. For both deletions, the distance between the residues linked by a glycine is compatible with the length of a 3 Cα atom segment in an extended conformation (about 6.5 Å). The linkage can therefore be expected to occur without distortion of the structure of the β barrel. *Panel B*, circular dichroism spectra (195 to 250 mm) of wild type aIF2γ (*black line*) and of aIF2γΔL1 (*gray line*). Spectra were recorded at 20 °C in a 1-mm path cuvette, using 2.5 μM of either protein in 10 mM potassium phosphate buffer, pH 6.7, containing 100 mM NaCl.

from deacylation. However, the dissociation constant of this ligand is increased by a factor of 3 compared with that of authentic Met-$tRNA_f^{Met}$ (Table I, rows 2 and 8). We conclude that the absence of strong base pairing at positions 1–72 as in *P. abyssi* $tRNA_i^{Met}$ or in *E. coli* $tRNA_f^{Met}$ contributes to optimal binding to aIF2. Finally, we observed that neither non-amino-

acylated $tRNA_f^{Met}$ (up to a concentration of 10 μM) nor free methionine (up to a concentration of 5 mM) competed with Met-$tRNA_f^{Met}$ binding to aIF2 (data not shown).

The α Subunit Reinforces the Binding of tRNA to the γ Subunit—The three-dimensional structure of the aIF2γ subunit shows a strong resemblance with EF-Tu:GTP. This similarity suggests a pivotal role of the aIF2γ subunit in the binding of tRNA. Accordingly, this subunit is active in the Gpp(NH)p-dependent protection of Met-$tRNA_f^{Met}$ against deacylation, although the corresponding dissociation constant (5 μM, Table II row 1, and Fig. 3) is markedly higher than that measured with the complete heterotrimeric aIF2 (120 nM; Table II, row 5). On the other hand, the binding is lost if Val-$tRNA_f^{Met}$ UAC is used as a ligand (Table II, row 2). Therefore, the γ subunit supports recognition of the methionyl group esterified on tRNA. In agreement with this idea, protection is restored by using the Met-$tRNA^{Val}$ CAU ligand (data not shown).

The large difference between the K_d value with the γ subunit and that with the full aIF2 trimer suggests that the α and/or the β subunit participate in tRNA binding. To test this idea, Met-$tRNA_f^{Met}$ dissociation constants were measured with either the αγ or βγ dimer. Whereas no improvement of the binding was observed with the βγ pair as compared with aIF2γ alone (Table II, rows 1 and 3), the affinity of Met-$tRNA_f^{Met}$ for αγ was similar to that of the intact trimer (Table II, rows 4 and 5).

By using aIF2α alone (up to 5 μM), no protection of Met-$tRNA_f^{Met}$ against deacylation could be observed. However, evidence that aIF2α interacts with tRNA was obtained from a gel shift experiment. As shown in Fig. 4 (*panel A*, *left*), the α subunit alone induces retardation of tRNA migration on a polyacrylamide gel. The apparent dissociation constant is on the order of 10 μM. Nevertheless, the RNA binding capacity of the isolated aIF2α subunit is not specific of the tRNA fold. Indeed, a mixture of 16 S and 23 S rRNA efficiently competed with the binding of tRNA to aIF2α. As shown in Fig. 4 (*panel B*, *left*), addition of ribosomal RNA in 2-fold excess (w/w) over tRNA abolishes the aIF2α-induced gel retardation of tRNA. These experiments show, at first sight, that aIF2α possesses general RNA binding properties that may account for the synergistic role of α in the binding of tRNA to the heterotrimer.

Mapping the α Subunit Binding Site onto aIF2γ—By analogy with tRNA bound to EF-Tu (41), a tRNA molecule can be docked onto aIF2γ (21). In this model, the 3′-end of the tRNA dips into a cavity formed by the β-strands of domain 2. Interestingly, this domain carries two elongated loops specific of the initiation factor (Fig. 1).

A possible role of these loops in the binding of either the α or β subunit was investigated using site-directed mutagenesis. Two deletion mutants of aIF2γ were constructed. The first deletion (γΔL1) corresponds to the replacement of residues 221–227 in loop 1 of domain 2 by a glycine. In the second mutant (γΔL2), residues 256–265 in loop 2 of domain 2 are replaced by a glycine. These two deletions were chosen to avoid perturbation of the structures of the mutants (Fig. 5). These two mutants were produced in large amounts in *E. coli,* were thermostable, and were purified with a yield of 20 mg of protein per liter of culture, as in the case of the wild-type aIF2γ protein. This argues in favor of a stable folding of the two proteins. The ability of the two mutants to associate with either the α or β subunit was tested by PAGE analysis under native conditions. The results in Fig. 6 (*panel A*, *lanes 8–10*) clearly show that the deletion of loop 2 in aIF2γ (γΔL2 mutant) has not changed the ability of the protein to bind the α and β subunits. In contrast, the γΔL1 mutant no longer binds the α subunit, whereas it has fully kept its capacity to bind the β (Fig. 6, *panel A*, *lanes 5–7*). Similar conclusions were reached using molecular sieving techniques (see "Experimental Procedures"). From these gel filtration experiments, assembly between subunits could be demonstrated unambiguously with the following combinations: βγ, αγ, αβγ, βγΔL1, βγΔL2, and αγΔL2. No assembly was observed with αβ (as previously observed in (21)) or with αγΔL1. With the αβγΔL1 combination, formation of the βγΔL1 dimer only was observed.

To assay the solution structure of aIF2γΔL1, the circular dichroism spectrum of the protein was compared with that of wild-type aIF2γ. As shown in Fig. 5 (*panel B*) the spectra of the two proteins are extremely similar. In addition, thermal unfolding of the two proteins was followed by monitoring the circular dichroism at 220 mm from 40 to 95 °C (protein concentrations of 15 μM in 10 mM potassium phosphate, pH 6.7, 100 mM NaCl; data not shown). Both purified proteins displayed identical unfolding curves, with a T_m of 85 °C in both cases. These observations strongly suggest that the ΔL1 mutation does not act by destroying the structure of domain 2. It can therefore be proposed that, among the features in domain 2 that distinguish archaeal and eukaryotic a/eIF2 from EF-Tu, the L1 loop is directly involved in the binding of the α subunit. Notably, during revision of the present manuscript, a study concerning aIF2γ from *M. jannaschii* was published on line (42). This study shows that aIF2γ-L256D (corresponding to Leu^{229} within loop 1 in *P. abyssi* aIF2, see Fig. 5) and aIF2γ-D325A (corresponding to D298 in *P. abyssi* aIF2) exhibited dramatic reductions in their aIF2α binding capacity. Asp-298 is located at the surface of domain 2, in close vicinity of loop 1. Our conclusion regarding the participation of loop 1 in the binding of aIF2α is therefore in good agreement with the results obtained in the case of *M. jannaschii* aIF2.

Domain 2 of aIF2γ Offers Crucial Determinants for Met-tRNA Binding—The L1 loop connects β7 and β8. Therefore, the γΔL1 mutant was also assayed for its ability to protect the ester bond in Met-$tRNA_f^{Met}$. As shown in Table II (row 6), protection against hydrolysis was no longer observed with up to 50 μM of this mutant. This behavior indicates that the dissociation constant of the complex containing the γΔL1 subunit and tRNA has been increased by at least 1 order of magnitude, as compared with the value obtained with the wild-type γ subunit (5 μM, Table II, row 1). In contrast, a trimer containing the γΔL2 mutant retains its full ability to protect Met-$tRNA_f^{Met}$ (Table II, row 8). Hence, the L1 loop, which is involved in the binding of the α subunit, appears to also participate in tRNA recognition. The observed effect of aIF2α on tRNA binding can therefore be due either to a direct interaction of aIF2α with tRNA or to a conformational adjustment of domain 2 of the γ subunit triggered by binding of the α subunit to the L1 loop.

In domain 2 of aIF2γ, a pocket formed by strands β7, β8, and β14 corresponds to the amino acid binding pocket in EF-Tu (21, 41) (Fig. 1). A signature sequence specific of all initiation factors (^{231}GGΦΦGG^{236}) is present in the β8 strand. Substitution by an aspartate of the third glycine in this signature sequence (G235D) does not modify the three-dimensional structure of the γ subunit, nor does it impair the capacity of γ to form an αβγ trimer (21). In the three-dimensional structure, the side chain of the substituting aspartate protrudes outside of the barrel. The trimer containing the aIF2γ-G235D mutated subunit was assayed for its ability to protect Met-$tRNA_f^{Met}$ against deacylation. As shown in Table II (row 9), protection was no longer obtained, even at a 50 μM concentration of the mutated trimer. This result implies that the affinity for Met-$tRNA_f^{Met}$ has decreased by at least 3 orders of magnitude compared with that of the intact trimer. Such data reinforce the idea that the pocket in domain 2 of aIF2γ indeed forms part of the methionine

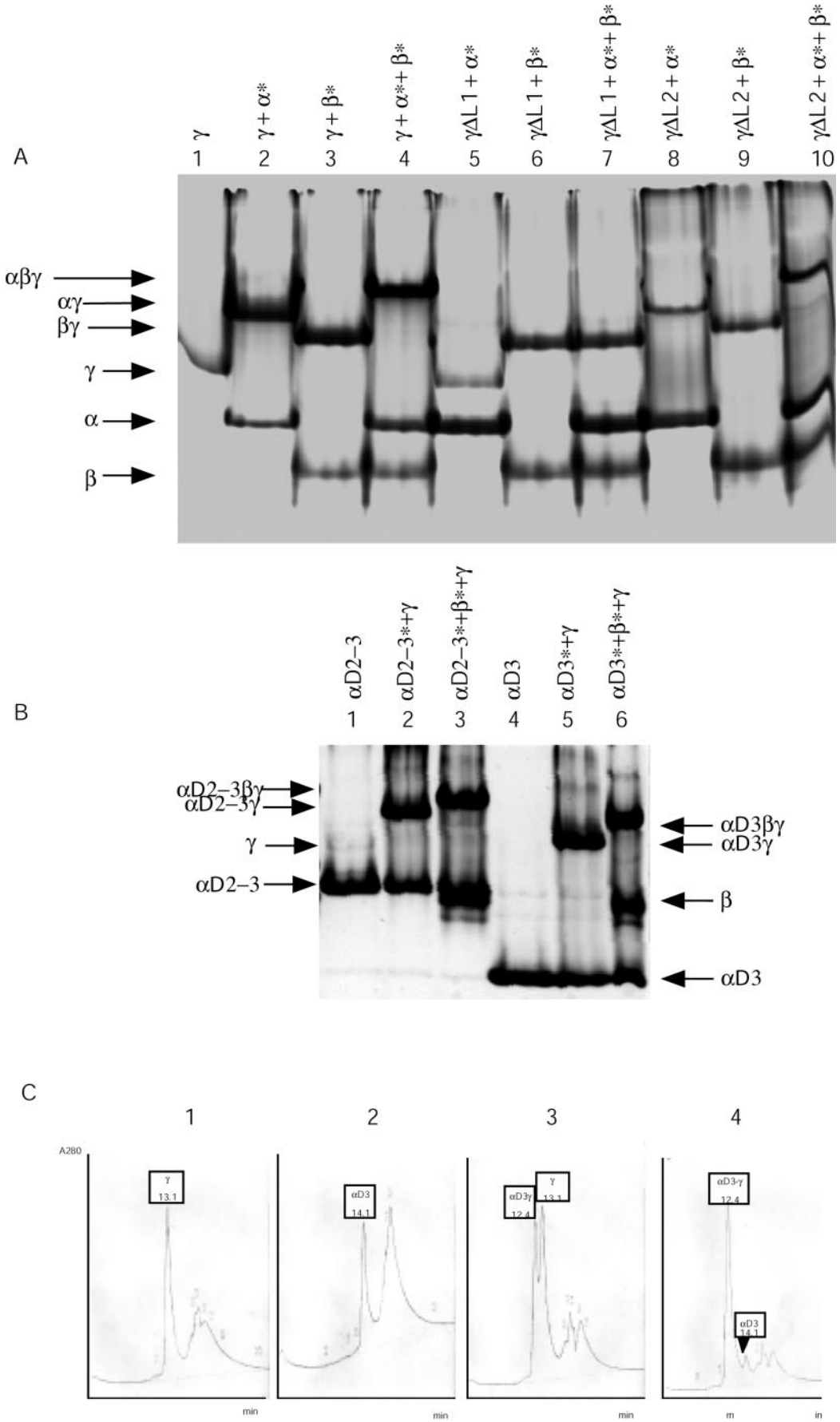

FIG. 6. **Assembly of aIF2 subunits and their mutants.** *Panels A* and *B*, prior to electrophoresis proteins were mixed at the final concentrations indicated below, in 10 μl of 200 mM NaCl, 10 mM MOPS, pH 6.7, and 10 mM β-mercaptoethanol. 10 μl of loading buffer (240 mM KOH, 144 mM acetic acid, 20% glycerol, 0.4% (w/v) methylene blue) was then added and the samples were applied onto a native acidic gel (see

binding site. The position of the Asp^{235} side chain outside the barrel (21) suggests that the mutation acts by steric hindrance and/or electrostatic repulsion.

In the Presence of Domain 3 of aIF2α, aIF2γ Exhibits Full tRNA Binding Capacity—The α subunit (314 amino acids) includes three domains. Domain 1 (residues 1–87) is an OB-fold, domain 2 (residues 88–172) is α helical, and the structure of domain 3 (residues 173–275) is not known (26). To identify the region in the α subunit that is anchored to the L1 loop, two truncated mutants of aIF2α were designed. Mutant αD1 (residues 1–87) contains domain 1. Mutant αD1–2 contains domains 1 and 2 (residues 1–172). These two mutants were produced in *E. coli* and purified. As shown by native PAGE analysis and molecular sieving, αD1 and αD1–2 do not bind aIF2γ. In addition, their presence does not increase the affinity of aIF2γ for tRNA (Table II, rows 10 and 11). Notwithstanding, gel shift experiments show that αD1 has the ability to interact with tRNA as well as with rRNA (Fig. 4, middle part of *panels A* and *B*).

To investigate an involvement of the third domain of aIF2α in the binding to aIF2γ, additional truncated forms of aIF2α were constructed. αD3 (residues 173–275) only contains the C-terminal domain of the α subunit. αD2–3 (residues 88–275) carries the central helical domain in addition to the C-terminal one. As shown by native PAGE and molecular sieving experiments (Fig. 6, *panels B* and *C*), these two truncated proteins were able to associate with aIF2γ. The dimer formed with αD3 plus γ was assayed for its ability to bind $Met\text{-}tRNA_f^{Met}$. As shown in Table II (rows 5 and 12), this dimer displays the same binding affinity for tRNA as the full aIF2 trimer. Domain 3 of aIF2α appears therefore to confer on aIF2γ full information for tRNA recognition. Finally, the RNA binding capacity of domain 3 was evaluated by using the gel shift assay. Fig. 4 shows that αD3 can bind either tRNA or rRNA (*right part* of *panels A* and *B*). This result raises the possibility that domain 3 contributes to complex formation between $Met\text{-}tRNA_f^{Met}$ and aIF2 through a direct interaction with the polynucleotide. However, it cannot be excluded that binding of αD3 to aIF2γ triggers a conformational adjustment of the tRNA binding site on the γ subunit.

DISCUSSION

Recognition of Initiator tRNA by aIF2—Various tRNAs aminoacylated with either valine or methionine were studied for their recognition by aIF2. The results demonstrate a strong preference of the protein for methionine over valine. The factor of discrimination is greater than 500. Moreover, esterification of two non-initiator tRNAs by methionine appears to be sufficient to confer on these tRNAs significant aIF2-binding properties. The key importance of the methionyl moiety in the recognition by aIF2 agrees with the early observation that misacylation of yeast initiator tRNA with isoleucine instead of methionine reduced by a factor of 400 the binding affinity of this tRNA for mammalian eIF2 (43).

Concerning the importance of the nucleotidic part of the ligand, several results have been obtained using eukaryotic systems. In particular, the base pair at the top of the 3′ acceptor end is known to be related to the initiator activity of eukaryotic tRNAs (44, 45). With rabbit eIF2, mutation of the $A^1\text{-}U^{72}$ base pair of human initiator Met-tRNA into a G-C one causes a loss of affinity by a factor of 10–17 (46). In *S. cerevisiae*, the activity in translation initiation of a mutant initiator tRNA with a $G^1\text{-}C^{72}$ base pair is markedly reduced *in vivo* (45). This mutation was very recently shown to cause a lowering by a factor of 7 of the affinity of an initiator tRNA transcript for yeast eIF2:GTP (47). In the present study, comparison of five methionylated tRNAs (Table I, rows 2–5 and 8) indicates that the binding by aIF2 is moderately sensitive to the base composition of the polynucleotidic ligand. In particular, the highly conserved $A^1\text{-}U^{72}$ base pair of archaeal initiator tRNAs is not strictly required for aIF2 recognition. Notwithstanding, a $G^1\text{-}C^{72}$ base pair, as encountered in most elongator tRNAs, disfavors the binding of tRNA. The discrimination factor between $Met\text{-}tRNA_i^{Met}$ ($A^1\text{-}U^{72}$) and $Met\text{-}tRNA^{Met}$ ($G^1\text{-}C^{72}$) is equal to 3. The minor importance of the nucleotidic moiety in the recognition process of $Met\text{-}tRNA_i^{Met}$ raises the question of the discrimination of elongator $tRNA^{Met}$ *versus* initiator in the archaeal cells. Several hypotheses can be proposed. (i) The archaeal elongator tRNA may contain specific features, such as modified bases, ensuring its rejection by aIF2. (ii) The *P. abyssi* initiator tRNA used in this study was produced in *E. coli*. It cannot therefore be excluded that the authentic tRNA has specific base modifications that might increase its affinity for aIF2. (iii) Finally, a factor of 3 may be sufficient to ensure preferential sequestering of $Met\text{-}tRNA_i^{Met}$ by aIF2. Moreover, $Met\text{-}tRNA^{Met}$ is a ligand of elongation factor aEF-1A. By reducing the concentration of free elongator tRNA, this binding should reduce the competition between the initiator and the elongator tRNAs toward binding to the aIF2 factor.

Role of the α Subunit—As previously shown (21), aIF2γ forms the structural core of the heterotrimeric aIF2 protein and has a three-dimensional structure markedly close to that of the active EF-Tu:GTP form (41). In the present study, we establish that aIF2γ specifically binds $Met\text{-}tRNA_i^{Met}$ in a GTP-dependent manner. The binding is cancelled by the G235D mutation that affects the third glycine in the $^{231}GG\Phi\Phi GG^{236}$ signature sequence. This sequence is inside the pocket formed by strands β7, β8, and β14. In EF-Tu, this pocket corresponds to the binding site of the esterified amino acid (41). These observations reinforce the idea that aIF2γ recognizes Met-tRNA in a manner similar to that of EF-Tu (21). However, the sequences of β7, β8, and β14 strands in initiation factors markedly differ from those in elongation factors. In bacterial EF-Tu, a threonine residue (Thr^{239}) replaces the Gly^{235} in aIF2γ. In *Caenorhabditis elegans* mitochondrial EF-Tu2, a Val (Val^{258}) is found at the position of this threonine. This valine possibly contributes to the strong affinity of EF-Tu2 toward Ser-tRNAs (48). Variations in the amino acid composition of the β7, β8, and β14

"Experimental Procedures"). After migration, the gels were stained with Coomassie Blue. *Panel A*, *lanes 1–4*, γ (12 μM, *lane 1*) mixed with molar excess of α (*lane 2*), β (*lane 3*), and α + β (*lane 4*). *Lanes 5–7*, γΔL1 (15 μM) mixed with molar excess of α (*lane 5*), β (*lane 6*), and α + β (*lane 7*). In each lane, the proteins in excess are indicated by *asterisks*. *Lanes 8–10*, γΔL2 (15 μM) mixed with molar excess of α (*lane 8*), β (*lane 9*), and α + β (*lane 10*). The *arrows* indicate the migrations of each subunit as well as those of the αγ and βγ dimers and that of the trimer (*lanes 1–4*). *Lanes 8–10* show that aIF2γΔL2 properly assembles with the two other subunits. *Lanes 5–7* show that aIF2γΔL1 assembles correctly with aIF2β but does not interact with aIF2α. *Panel B, lanes 1–3*, αD2–3 (34 μM) (*lane 1*), mixed with γ (18 μM) (*lane 2*), or with γ (18 μM) plus β (25 μM) (*lane 3*). In each lane, the proteins in excess are indicated by *asterisks*. *Lanes 4–6*, αD3 (54 μM) (*lane 1*), mixed with γ (18 μM) (*lane 5*), or with γ (18 μM) plus β (25 μM) (*lane 6*). The same experiments were performed with αD1 and αD1–2. No binding of these truncated α subunits to the γ subunit could be evidenced (gels not shown). Migrations of isolated β and γ subunits were assayed on the same gel. Their positions are shown by *arrows*. The band corresponding to an αD3γ dimer (*lane 5*) was cut and the proteins were extracted. Further gel analysis of the sample onto a denaturing 16% acrylamide gel evidenced stoichiometric amounts of γ and αD3. *Panel C*, size exclusion chromatography assay for the assembly of aIF2γ with αD3. Samples were injected onto a TSK3000 SWXL column (Tosohaas, Japan) as described under "Experimental Procedures." The shown chromatograms correspond to the following injection conditions: *1*, 100 μl of 12 μM aIF2γ; *2*, 100 μl of 17 μM αD3; *3*, 100 μl of 17 μM aIF2γ plus 8 μM αD3; *4*, 100 μl of 12 μM aIF2γ plus 25 μM αD3. Measured retention times are indicated in *boxes*.

strands may therefore explain the relative specificity of each factor toward the various aminoacyl moieties attached to tRNAs.

The heterotrimer aIF2 displays, however, much more affinity toward its ligand than does aIF2γ alone. We show that, under our assay conditions, aIF2β does not contribute to the reinforcement of the binding of tRNA. On the other hand, addition of aIF2α to aIF2γ increases the binding affinity by 2 orders of magnitude. Recognition between the two subunits requires an idiosyncratic extended loop of aIF2γ (Fig. 1), on the one side, and domain 3 of aIF2α, on the other side. Actually, domain 3 alone is enough to confer on aIF2γ the same tRNA binding affinity as that displayed by the full heterotrimer.

Up to now, implication of eukaryotic eIF2α in the regulation of nucleotide exchange has retained the most attention. Indeed, eukaryotic cells contain eIF2B, a five-subunit nucleotide exchange factor in which the ϵ subunit plays the catalytic role (49). Moreover, eukaryotic eIF2α responds to stress conditions by undergoing phosphorylation of a strictly conserved serine at position 51. Upon this modification, eIF2 acquires the capacity to sequester eIF2B thereby blocking the nucleotide exchange activity in the cell (reviewed in Refs. 27–30). The archaeal α subunit rarely shows an equivalent of serine 51. Furthermore, in Archaea, orthologs of 3 subunits of eIF2B (α, β, δ) could be identified, but the γ and ϵ subunits have no equivalent (18). Therefore, in Archaea, nucleotide exchange on aIF2 is likely to occur spontaneously. These characteristics may explain different functions of aIF2α in Eukarya and in Archaea, respectively. Nevertheless, in view of the present results, the possibility that, in Eukarya, the α subunit also plays a role in tRNA binding deserves some discussion.

The importance of the function of eIF2α in *S. cerevisiae* was first evidenced from the demonstration that mutations in the gene of this protein (*SUI2*), corresponding to amino acid changes in the N-terminal domain, conferred a haploid strain the ability to initiate translation at *HIS4* on a UUG start codon (32). This indicated that eIF2α was important for the accuracy of selection of the start codon. Moreover, the *SUI2* gene is essential for yeast viability. However, yeast null mutants for *SUI2* could recover the capacity to grow slowly upon overexpression of both eIF2$\beta\gamma$ and initiator tRNA (50). After purification from this strain, by using a nitrocellulose binding assay, the $\beta\gamma$ dimer was shown to form a complex with Met-tRNA$_i^{Met}$. The corresponding K_d value is increased by a factor of 5 as compared with the K_d value measured with the intact trimer (31). This indicates a positive contribution of the α subunit to tRNA binding. Nevertheless, because of large standard errors in the measurements, this conclusion was considered cautiously by the authors. In fact, the observation that overexpression of both the $\beta\gamma$ dimer and the initiator tRNA partly rescues the growth of a yeast strain deprived of aIF2α, indirectly argues in favor of a positive role of the α subunit in tRNA binding by eIF2. Indeed, by increasing the fraction of complexed tRNA available for translation initiation, larger amounts of the $\beta\gamma$ dimer and of initiator tRNA may compensate for the decreased affinity of tRNA for the $\beta\gamma$ dimer as compared with the trimer.

Functional Implications—Domain 3 of aIF2α is fully responsible for the reinforcement of complex formation between initiator tRNA and aIF2γ. Indeed, domains 1 and 2 of aIF2α appear to play no role in Met-tRNA$_i^{Met}$ binding. However, domain 1 of α displays general RNA binding properties. Actually, this domain 1 is a β barrel resembling bacterial initiation factor IF1 (26, 51). Interestingly, IF1 recognizes the 16 S rRNA inside the ribosomal A-site (52, 53). It can be therefore imagined that, whereas domain 3 of aIF2α is bound to the γ subunit, domain 1 of aIF2α anchors aIF2 to the ribosome. Another possibility is a contact of domain 1 with the mRNA. Whatever the case, a binding of aIF2α to either rRNA or mRNA might support an involvement of this subunit in start codon selection. Indeed, mutations within domain 1 of yeast eIF2α are known to cause a relaxed start codon specificity (32).

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