Conserved but nonessential interaction of SRP RNA with translation factor EF-G

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ABSTRACT

4.5S RNA is essential for viability of *Escherichia coli*, and forms a key component of the signal recognition particle (SRP), a ubiquitous ribonucleoprotein complex responsible for cotranslational targeting of secretory proteins. 4.5S RNA also binds independently to elongation factor G (EF-G), a five-domain GTPase that catalyzes the translocation step during protein biosynthesis on the ribosome. Point mutations in EF-G suppress deleterious effects of 4.5S RNA depletion, as do mutations in the EF-G binding site within ribosomal RNA, suggesting that 4.5S RNA might play a critical role in ribosome function in addition to its role in SRP. Here we show that 4.5S RNA and EF-G form a phylogenetically conserved, low-affinity but highly specific complex involving sequence elements required for 4.5S binding to its cognate SRP protein, Ffh. Mutational analysis indicates that the same molecular structure of 4.5S RNA is recognized in each case. Surprisingly, however, the suppressor mutant forms of EF-G bind very weakly or undetectably to 4.5S RNA, implying that cells can survive 4.5S RNA depletion by decreasing the affinity between 4.5S RNA and the translational machinery. These data suggest that SRP function is the essential role of 4.5S RNA in bacteria.

Keywords: SRP; 4.5S RNA; EF-G; SRP RNA

INTRODUCTION

The signal recognition particle (SRP) is a cytosolic ribonucleoprotein that mediates cotranslational targeting of secretory and membrane proteins to the endoplasmic reticulum or the plasma membrane in eukaryotes or bacteria, respectively (for review, see Keenan et al. 2001; Nagai et al. 2003). Mammalian SRP consists of a 7S RNA molecule and six proteins, SRP72, -68, -54, -19, -14, and -9. The SRP54 protein, in complex with the phylogenetically conserved domain IV of SRP RNA, forms the functional SRP core that recognizes signal sequences, interacts with the SRP receptor, and binds and hydrolyzes GTP (Bernstein et al. 1989; Romisch et al. 1989). In *Escherichia coli*, the SRP is composed of the Ffh protein, a 48 kD homolog of SRP54, bound to the 4.5S RNA. 4.5S RNA, encoded by the essential gene *ffs* (Brown and Fournier 1984), is a metabolically stable, 114 nucleotide bacterial homolog of the 7S RNA that includes the conserved domain IV region containing the binding site for SRP54/Ffh. Depletion of 4.5S RNA in *E. coli* leads to defects in secretion of several classes of proteins, consistent with the established role of 4.5S RNA in the function of SRP.

At the same time, several lines of evidence have suggested that 4.5S RNA is also involved in protein synthesis on the ribosome. The typical *E. coli* cell requires ~1000 molecules of 4.5S RNA to maintain growth, a ~twofold excess over the amount of Ffh in the cell (Phillips and Silhavy 1992; Jensen et al. 1994). The discovery that point mutants of the protein synthesis elongation factor G (EF-G) suppress the effect of 4.5S RNA depletion (Brown 1987) suggested that 4.5S RNA plays a direct role in translation. EF-G catalyzes translocation of the mRNA–tRNA complex during protein synthesis, advancing the translating ribosome along mRNA by one codon and opening the ribosomal A site for the next incoming tRNA (for review, see Joseph 2003). During each cycle of translocation, EF-G bound GTP is cleaved to GDP and phosphate, and EF-G—GDP dissociates from the ribosome before the next amino acid is incorporated (Winter-

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meyer and Rodnina 2000). However, slow translocation occurs in the absence of EF-G, indicating that EF-G accelerates protein synthesis by reducing the activation barrier that separates the pre- and post-translocational states of the ribosome (Cukras et al. 2003). Mutagenesis and in vitro binding assays showed that EF-G binds directly to 4.5S RNA in a region containing a decanucleotide sequence conserved in the EF-G binding site in 23S ribosomal RNA, supporting the idea that 4.5S RNA might be specifically involved in the translocation step of polypeptide synthesis (Shibata et al. 1996; Nakamura et al. 1999, 2001).

Brown (1987) originally hypothesized that EF-G mutations might suppress 4.5S RNA depletion by sequestering 4.5S RNA on the ribosome, conserving its ribosomal function over its role in SRP in the event of limiting 4.5S RNA. The observation that most of the 4.5S RNA colocalizes with ribosomes in one of the EF-G suppressor mutant strains seemed to support the idea that the ribosome might be the critical site of 4.5S RNA action (Brown 1989; Gu et al. 2003). However, sequencing of the two identified mutations in EF-G revealed that both are Leu → Gln mutations (L221Q and L262Q) located in the hydrophobic core of the G′ subdomain of the protein (Johanson et al. 1996). How these point changes might affect 4.5S RNA binding was not obvious, given that the G′ domain does not share electrostatic or structural features common to known RNA binding motifs (AEvarsson et al. 1994; Czworkowski et al. 1994). Nonetheless, the mutations are located on the surface of EF-G when the protein is bound to the ribosome, consistent with the possibility of a direct interaction with 4.5S RNA (Agrawal et al. 1998; Valle et al. 2003).

To address the mechanism of the EF-G suppressor mutants and the corresponding role of 4.5S RNA in translation, we tested the binding affinity and specificity of the wild-type and suppressor mutant EF-G proteins for 4.5S RNA. We show that 4.5S RNA and wild-type EF-G form a low-affinity but highly specific complex involving structural elements required for 4.5S binding to its cognate SRP protein, Ffh. Furthermore, 4.5S RNA binds with similar specificity to *Methanococcoides burtonii* EF2, an archaeal EF-G homolog, demonstrating that this interaction is phylogenetically conserved. Surprisingly, however, the suppressor mutant forms of EF-G bind very weakly or undetectably to 4.5S RNA, implying that reduced affinity between 4.5S RNA and the translational machinery enables cells to survive when 4.5S RNA is in short supply. These data suggest that SRP function is the essential role of 4.5S RNA in bacteria.

RESULTS

Wild-type EF-G binds 4.5S RNA in a low-affinity, highly specific complex

The conserved domain IV of SRP RNA, containing the binding site for the Ffh protein, includes a 10-nucleotide sequence identical to a region in 23S ribosomal RNA, the sarcin-ricin loop, implicated in EF-G binding (Nakamura et al. 1999, 2001; Fig. 1A). However, this same decanucleotide sequence forms entirely different structures in 4.5S RNA versus 23S rRNA (Conn et al. 1999; Schmitz et al. 1999; Wimberly et al. 1999; Ban et al. 2000; Batey et al. 2000; Jovine et al. 2000), raising the possibility that EF-G recognizes a different 4.5S RNA structure than that bound by Ffh in the SRP complex. To test this idea, the affinity and specificity of the interaction between EF-G and 4.5S RNA was investigated using purified full-length wild-type EF-G protein and a series of wild-type or mutant forms of the 4.5S RNA. Equilibrium binding constants were determined using a nitrocellulose filter binding assay (Fig. 2; Table 1). The binding constant of 4.5S RNA and EF-G is ≈ 2 μM, an interaction affinity that was not significantly affected by changes in the second and third nucleotides of the tetraloop (Figs. 1, 2; Table 1). Moderate (four- to eightfold) effects were observed when the tetraloop sequence was completely changed, while changes in the base pairing sequence or stem length adjacent to the tetraloop resulted in 10- to 18-fold effects in the measured dissociation constant. Deletion or insertion of one or two base pairs adjacent to the tetraloop (constructs ST + 1, ST − 1, ST + 2, and ST − 2, Figs. 1B, 2) caused a decrease in binding affinity for EF-G beyond the

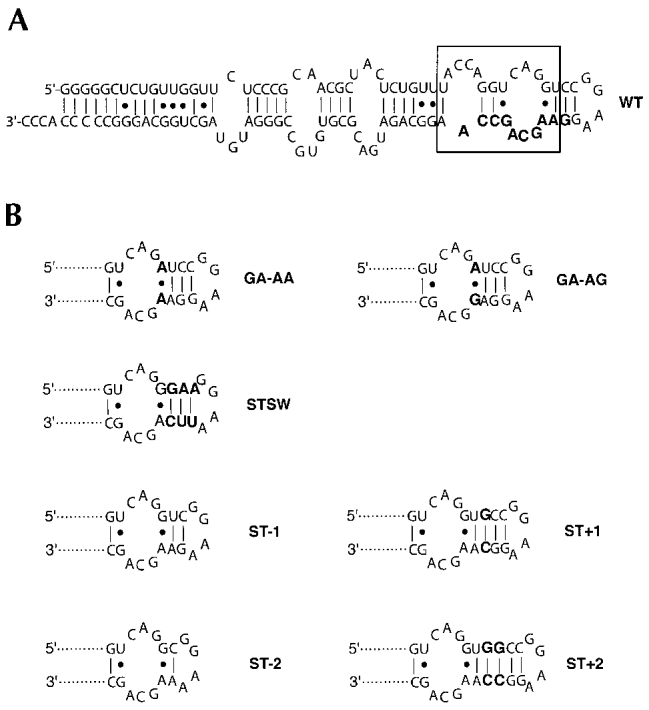

FIGURE 1. (*A*) Predicted secondary structure and nucleotide sequence of *E. coli* 4.5S RNA. The bold region marks the conserved decanucleotide sequence identical in 4.5S and 23S RNA. The part of the RNA recognized by the Ffh M domain is boxed. (*B*) Secondary structures of *E. coli* mutant 4.5S RNA constructs. Although only the mutated portion of each RNA is shown, mutations were made within the full-length 4.5S RNA.

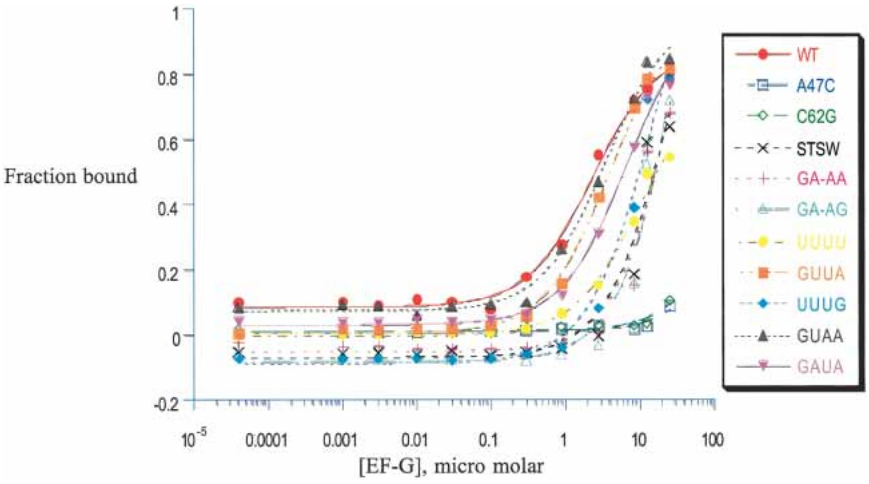

FIGURE 2. Representative binding curves for wild-type 4.5S RNA (red circles) and several mutants (A47C, blue squares; C62G, green diamonds; STSW, crosses; GA-AA, plus signs; GA-AG, empty triangles; UUUU, yellow circles; GUUA, red squares; UUUG, filled diamonds; GUAA, filled triangles; GAUA, reverse triangles).

limit of detection (>100 μM). Similarly, point mutations in the symmetric internal loop (A47C and C62G) decreased binding affinity for EF-G beyond the detection limit, as observed previously (Nakamura et al. 2001). These mutations, which disrupt part of the 4.5S RNA structure observed in the SRP signal binding domain (Batey et al. 2000), also cause a complete loss of detectable binding to the Ffh protein. These data are consistent with qualitative binding studies using a truncated form of EF-G (Nakamura et al. 2001).

EF-G-catalyzed translocation of peptidyl tRNAs from the ribosomal A site to the P site is a GTP-dependent process, and structural studies suggest that significant conformational changes occur in EF-G, depending on its nucleotide-bound state (GTP vs. GDP; AEvarsson et al. 1994; Czworkowski et al. 1994). To test whether nucleotide binding would affect the interaction of EF-G with 4.5S RNA, binding assays with wild-type EF-G and 4.5S RNA were repeated in the presence of a saturating concentration of either GTP or GDP. Although GTP did not affect binding affinity detectably, a twofold increase in affinity occurred in the presence of GDP, consistent with previous results (Shibata et al. 1996; data not shown).

TABLE 1. Binding characteristics of full-length wild-type EF-G with 4.5S RNA

Mutation	K_d (μM)	$\Delta\Delta G$
4.5S RNA, wild type	2.3 ± 1.0	n.a.
Tetra-loop mutants		
UUUU	11.1 ± 1.6	0.9
GUUA	6.3 ± 0.4	0.56
UUCG	18.0 ± 0.7	1.2
GUAA	6.1 ± 0.4	0.5
GAUA	9.0 ± 1.3	0.8
Stem-loop mutants		
GA-AA	37.1 ± 5.5	1.6
GA-AG	44.3 ± 2.9	1.7
Stem-switch	25.3 ± 2.3	1.5

A47C, C62G, ST+1, ST−1, ST+2, and ST−2 abolished detectable interaction with full-length wild-type EF-G.

Interaction between 4.5S RNA and EF-G is phylogenetically conserved

The sequence of domain IV of SRP RNAs is conserved in all three kingdoms of life (Fig. 1A). Likewise, homologs of EF-G are found in archaea and eukaryotes, raising the possibility that the 4.5S-EF-G interaction is phylogenetically conserved as well. To test this idea, we purified the EF-G homolog, aEF2, from the archaeon *M. burtonii* and tested its binding affinity and specificity for *E. coli* 4.5S RNA. The affinity of this interaction was found to be approximately 10-fold weaker than that observed with EF-G (~20 μM; Table 2). Although a similar affinity was observed with the tetraloop (GAAA) mutant, mutations A47C and C62G (Fig. 1A) decreased binding affinity to aEF2 beyond detectable levels. Deletion of one or insertion of one or two base pairs adjacent to the tetraloop (constructs ST − 1, ST + 1, and ST + 2; Fig. 1B) also caused a significant loss of binding affinity (Table 2). The binding characteristics thus show a similar pattern of interaction to that observed in the bacterial counterpart involving EF-G and 4.5S RNA.

Loss of 4.5S RNA binding affinity by EF-G mutants that suppress 4.5S RNA depletion

The mutations L221Q and L262Q occur within the hydrophobic core of the G′ subdomain, a region of EF-G suggested to be an internal guanine-exchange factor (AEvarsson et al. 1994; Czworkowski et al. 1994). Both of these amino acid side chains pack against residues that form a deep pocket in the protein surface, implying that the mu-

TABLE 2. Binding characteristics if EF2 with 4.5S RNA

Mutation	K_d (μM)	$\Delta\Delta G$
4.5S RNA, wild type	22.6 ± 2.3	n.a.
Tetra-loop mutants		
GAAA	16.0 ± 1.4	−0.2
Stem-loop mutants		
ST+1	>>50	>>0.6
ST−1	>>50	>>0.6
ST+2	>>50	>>0.6

A47C and C62G abolished detectable interaction with EF2.

tations will have a similar effect on the G′ subdomain structure. Although this cleft may constitute a ligand binding site, the very acidic nature of the G′ subdomain makes it unlikely to be a direct binding site for RNA. To test this, the two mutant forms of EF-G were cloned, overexpressed in *E. coli*, and purified. Multiple-turnover GTP hydrolysis assays were performed, demonstrating that each mutant protein retained GTPase activity comparable to that observed in the wild-type EF-G (data not shown). To examine the affinity and specificity of the interaction of each mutant EF-G protein with the 4.5S RNA, equilibrium dissociation constants were measured by filter binding. Surprisingly, the L221Q mutation in EF-G essentially abolished the ability to bind the 4.5S RNA (Fig. 3). The L262Q mutation in EF-G resulted in a ~20-fold decrease in binding affinity for 4.5S RNA (Fig. 3). Binding affinities in each case were unaltered in the presence of a saturating concentration of GTP.

DISCUSSION

Genetic and biochemical evidence have supported a dual role for 4.5S RNA in bacteria, both as part of the signal recognition particle and as an aid to translocation of polypeptides during protein synthesis on the ribosome. Although 4.5S RNA is essential in *E. coli* (Phillips and Silhavy 1992) and other bacteria (i.e., *Bacillus subtilis*; Nakamura et al. 1992), it has been unclear which of its functions is required for cell viability. A truncated form of the 4.5S RNA spanning the phylogenetically conserved domain IV stem–loop supports growth of an *E. coli* strain depleted of endogenous 4.5S RNA (Batey et al. 2000). Similarly, *E. coli* 4.5S RNA or a shortened form of *B. subtilis* SRP RNA corresponding to domain IV will functionally compensate for the loss of endogenous SRP RNA in *B. subtilis* cells (Nakamura et al. 1992). Domain IV includes the binding site for the SRP protein Ffh, responsible for GTP-dependent signal peptide recognition. Domain IV has also been shown to bind the protein synthesis translocation factor EF-G, and contains a 10-nucleotide sequence identical to that of the α-sarcin/ricin EF-G binding region of 23S ribosomal RNA. This functional overlap within the same region of 4.5S RNA might imply similar structures of domain IV of 4.5S RNA and the α-sarcin/ricin loop. However, comparison of the crystal structures of the bacterial small ribosomal subunit with the crystal structure of the ribonucleoprotein core of the signal recognition particle shows significant differences in the conformation of the decanucleotide sequence.

In an effort to determine whether both the SRP and ribosomal functions of 4.5S RNA are evolutionarily conserved, and to elucidate the essential role of the RNA in bacteria, we studied the affinity and specificity of its interaction with EF-G. Wild-type EF-G forms a low-affinity but high-specificity complex with 4.5S RNA in an interaction that is sensitive to mutations known to affect both RNA structure and binding of the Ffh protein. These data suggest that the EF-G protein recognizes the same structure that is bound by Ffh, although with ~1000-fold weaker affinity.

Furthermore, the interaction between EF-G and 4.5S RNA appears to be evolutionarily conserved, based on the observation that an archaeal homolog of EF-G, *M. burtonii* aEF2, also binds weakly but with similar relative specificity to 4.5S RNA. This result is consistent with the previous observation that *B. subtilis* EF-G binds to 4.5S RNA from both *E. coli* and *Clostridium perfringens* (Shibata et al. 1996). Interestingly, the sequence conservation between the archaeal and bacterial proteins is only ~30%, with the highest conservation in the G domain responsible for GTP binding and hydrolysis (Cammarano et al. 1992; Creti et al. 1994; Thomas and Cavicchioli 1998). In contrast, the sequence of domain IV of 4.5S RNA is highly conserved in all three kingdoms of life, supporting the idea that this part of the RNA arose early in evolution, and that its sequence is constrained by multiple interacting factors.

Mutations in EF-G, as well as in 23S rRNA in the region spanning the EF-G binding site, reduce the requirement of 4.5S RNA by severalfold, providing the original genetic evidence for a role of 4.5S RNA in translocation of polypeptides on the ribosome (Brown 1987). In contrast to one prediction of this hypothesis, however, we find that the mutant proteins bind weakly or undetectably to 4.5S RNA. Each mutant protein contains a single amino acid change within the hydrophobic core of the G′ subdomain, adjacent to the GTPase domain in the structure. The G′ domain is not conserved, at least at the sequence level, among EF2 homologs of EF-G, and it also appears unlikely to be an RNA binding site based on its acidic property (Johanson

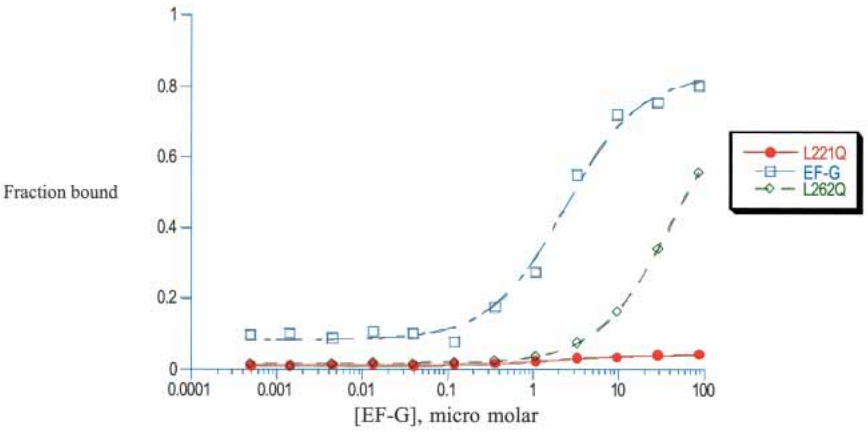

FIGURE 3. Comparison of binding affinity of full-length wild-type EF-G (squares) with the mutants (L221Q, circles; L262Q, diamonds). Representative binding curves are shown.

et al. 1996). Thus, the suppressor mutations probably indirectly affect RNA binding by affecting the conformation of the EF-G protein. Because the GTPase activity of each mutant protein was similar to that of the wild-type EF-G, and because GTP and GDP had minimal effects on 4.5S binding affinity in both the wild-type and mutant EF-G proteins, any conformational change induced by the suppressor mutations appears to be independent of the GTP-bound state of the protein, at least in vitro.

Our data indicate that the same conformation of 4.5S RNA observed in the signal recognition particle is recognized by EF-G, based on the fact that mutations in the 4.5S RNA have similar relative effects on binding affinity for both Ffh and EF-G (Batey et al. 2001). These results are inconsistent with the hypothesis that 4.5S RNA can substitute for the α-sarcin/ricin region of 23S rRNA on the ribosome and compete for EF-G binding, as has been proposed. Instead, our data suggest a model in which 4.5S RNA forms a single structure recognized by both Ffh and EF-G proteins, where the lower affinity of the EF-G complex ensures that sufficient 4.5S RNA is always available for Ffh binding, and consequently for SRP function. The EF-G suppressor mutations weaken affinity for 4.5S RNA binding even further, suppressing the effects of 4.5S RNA depletion by ensuring that sufficient RNA is available for SRP activity. Based on these data, the role of 4.5S RNA in translation is apparently dispensable.

This model is consistent with several previous observations regarding the activity and localization of 4.5S RNA. Only a small fraction of 4.5S RNA is associated with ribosomes in wild-type cells (Lee et al. 1978), and the sensitivity of this interaction to the antibiotic puromycin, which prematurely terminates the growing polypeptide chain (Brown 1987; Nakamura et al. 1999), indicates that 4.5S RNA acts transiently at the ribosome rather than as a stable component of a small subset of ribosomes. In wild-type *E. coli*, 4.5S RNA comprises approximately 2% of the tRNA content (Hsu et al. 1984), representing ~3000 molecules of 4.5S RNA per cell (Neidhardt 1987). Ffh–4.5S RNA complexes account for just 20% of the total cellular 4.5S RNA (Suzuma et al. 1999); although the majority of 4.5S RNA might therefore be available for EF-G binding, the intracellular concentration of EF-G (~10-fold above that of Ffh (Jensen et al. 1994) is insufficient for stable EF-G–4.5S RNA association in vivo. One puzzling observation, in light of our EF-G binding data, is the finding that in the mutant *E. coli* strain carrying an EF-G L221Q mutation (sffA; Brown 1987), 4.5S RNA colocalizes with the ribosome. This does not occur with the L262Q mutant strain, suggesting the possibility that the two mutants suppress 4.5S RNA depletion by different mechanisms, perhaps involving other as yet unidentified factors. Although the function of SRP RNA on the ribosome is dispensable, this activity appears to have been conserved in evolution. Most available evidence supports a role for 4.5S RNA in the elongation step of translation, in which peptidyl-tRNA moves from the aminoacyl-tRNA (A site) to the peptidyl-tRNA site (P site) following peptide bond formation on the ribosome, although the possible participation of 4.5S RNA in translation initiation has also been suggested (Bourgaize and Fournier 1987). Whether 4.5S RNA can be directly linked to the translocation step of protein synthesis will require analysis using a single-turnover assay in which the translocation step can be isolated.

MATERIALS AND METHODS

Cloning of wild-type EF-G and the suppressor mutants L221Q and L262Q

A vector for the expression of full-length wild type EF-G was constructed using standard molecular cloning techniques. Two oligonucleotide primers were designed to create NdeI and BamH1 restriction sites at the 5′ and 3′ ends of the gene, respectively, as well as a hexahistidine tag followed by a tobacco etch virus (TEV) protease cleavage site at the amino terminus of the encoded protein sequence. These primers were used in a polymerase chain reaction (PCR) with Vent DNA polymerase (New England Biolabs) to amplify the EF-G gene from the plasmid DNA of a construct generously provided by Kevin Wilson using a standard amplification protocol. The PCR reaction was subsequently digested with NdeI and BamH1 restriction enzymes, and purified on a 1% agarose gel. The band corresponding to the insert was excised from the gel and purified using a QIAEX purification kit (Qiagen). The product was ligated into a pET15b vector (Novagen) linearized with NdeI and BamH1 using T4 DNA ligase and incubated at 16°C for 4 h. The ligation reaction was used to transform *E. coli* DH5α (Gibco-BRL). Transformants were selected on the basis of ampicillin resistance, and the plasmids were sequenced to confirm the correct sequence. The L221Q and L262Q mutations were introduced into the full-length wild-type *E. coli* EF-G using mutagenic oligonucleotides in a recombinant PCR reaction.

Expression and purification of wild-type EF-G and the mutants L221Q and L262Q

Plasmids encoding the wild-type EF-G and the mutants L221Q and L262Q EF-G were transformed into *E. coli* BL21(DE3)/pLysS (Novagen) cells by heat shock. Freshly transformed cells were grown in Luria Broth at 37°C for the wild-type construct, and in CSB media (0.5% glucose, 0.1% cassamino acids, 0.5 mM $MgSO_4$, 0.01 mg/mL Thiamine, 0.025% HCl containing 0.005% trace metal) at 20°C for the mutant constructs, in the presence of 100 μg/mL ampicillin and 67 μg/mL chloramphenicol. When the absorbance at 600 nm reached 0.8, protein expression was induced with IPTG (1 mM for the wild-type EF-G, 0.6 mM for both mutant proteins). Pelleted cells were lysed by sonication at 4°C (5–10 × 1-min periods) in buffer A (50 mM Tris-HCl, pH 8.0/100 mM NaCl/1 mM EDTA/10 mM β-mercaptoethanol). After centrifugation, the supernatant was loaded onto a Ni-NTA agarose (Qiagen) column preequilibrated with buffer B (20 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol, 100 mM NaCl, 10% glycerol, 5 mM imidazole). The column was washed with five column volumes of buffer B containing 10 mM imidazole to avoid nonspecific

binding. The protein was eluted with 10 column volumes of buffer B containing 400 mM imidazole. Fractions containing protein were identified by nonreducing polyacrylamide gel electrophoresis. To remove the hexahistidine tag, 30 mg of N-His_6-EF-G was resuspended with approximately 2500 units of recombinant TEV protease (TEVP) and incubated overnight at 4°C while dialyzing in 1 liter of buffer C (20 mM Tris-HCl, pH 8.0, 10% glycerol, 2 mM DTT, 0.2 mM EDTA). Another round of Ni-NTA column chromatography was carried out to remove the cleaved hexahistidine tag and TEVP. The cleaved protein was dialyzed into buffer C and loaded onto a Mono-Q column (Pharmacia) equilibrated into the same buffer. After elution using a 20-column volume linear gradient to 100% buffer D (buffer C and 1 M NaCl), the protein was dialyzed into a storage buffer with 20 mM Tris-HCl, pH 8.0, 2 mM DTT, and 100 mM NaCl.

The activity of the purified wild-type EF-G and the L221Q and L262Q EF-G mutant proteins was confirmed using a GTP-hydrolysis assay as described (Johanson et al. 1996). The respective K_{cat} values are 1.046, 1.0812, and 1.5908 sec^{-1}.

Expression and purification of archaeal EF2

The plasmid containing the *M. burtonii* Elongation Factor 2 (EF2), provided by Dr. Richardo Cavicchioli (University of New South Wales, Australia), was transformed into *E. coli* BL21 Codon Plus-RIL competent cells (Stratagene) by heat shock. Freshly transformed cells were grown in Luria Broth media supplemented with 100 μg/mL ampicillin and 67 μg/mL chloramphenicol at 37°C until the absorbance at 600 nm reached 0.8; protein expression was induced by addition of 1 mM IPTG. Pelleted cells were lysed by sonication at 4°C (5–10 × 1-min periods) in buffer A (50 mM Tris-HCl, pH 8.0/100 mM NaCl /1 mM EDTA/10 mM β-mercaptoethanol), and EF2 was purified through a chitin column followed by DTT-induced intein-mediated self-cleavage of the fusion protein (Thomas and Cavicchioli 1998).

Equilibrium binding assays

Complexes of wild-type or mutant EF-G with 4.5S RNA were detected and quantitated using a nitrocellulose filter-binding assay (Wong and Lohman 1993). A constant concentration of 5′-^{32}P-end labeled RNA (~1 pM) was incubated in a buffer containing 20 mM Tris-HEPES (pH 7.5), 200 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.01% Igepal C-680 and 0.1 mg/mL tRNA with varying concentrations of protein. End-labeled RNA was denatured and renatured by heating to 90°C for 90 sec and cooling on ice for 4 min in TE buffer prior to use. Binding was found to be independent of tRNA concentration. The 100-μL reactions were incubated at room temperature for 1 h prior to application to filters containing three membranes: a 0.45-μm filter to retain any aggregate, a BA-85 nitrocellulose membrane (Schleicher and Schull) to retain protein–RNA complexes, and a Hybond-N+ (Amersham) positively charged nylon membrane to retain free RNA. These filters were soaked in wash buffer (20 mM Tris-HEPES, pH 7.5, 200 mM KCl, 10 mM $MgCl_2$) for 1 h prior to being placed within a 96-well dot blot apparatus. Aliquots (40 μL) from each reaction were sequentially added to the wells, while vacuum was being applied, followed by washing with 100 μL of wash buffer. The filters were allowed to air dry for 30 min. Free and bound RNA retained on the filters was detected using a Molecular Dynamics phosphorimager and quantitated using ImageQuant (Molecular Dynamics). Data were analyzed using nonlinear least-square analysis using KaleidaGraph and Microsoft Excel. Background intensities (<10% of the total intensity of each spot) were subtracted from the intensities of all spots. The fraction bound was then calculated to be the intensity of the spot on the nitrocellulose filter divided by the sum of the intensities on the spots on the nitrocellulose and Hybond-N+ filters. A least-squares fit for a single binding site was obtained using the equation

$$f = (a-b)\ [P/(P + K_d)] + b,$$

where a is the maximum fraction bound at saturating EF-G, b is the amount theoretically bound in the absence of EF-G, P is the active EF-G concentration, and K_d is the apparent equilibrium dissociation constant. All K_d and $\Delta\Delta G$ values reported are the average values from at least three independent experiments.

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