Minimalist Aminoacylated RNAs as Efficient Substrates for Elongation Factor Tu†

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ABSTRACT: We demonstrate here, using RNA variants derived from $tRNA^{Asp}$, that the minimalist aminoacylated structure able to interact efficiently with elongation factor Tu comprises a 10 base-pair helix linked to the 3′-terminal NCCA sequence. Shorter structures can interact with the elongation factor, but with significantly decreased affinity. Conserved features in the aminoacyl acceptor branch of tRNAs, such as base pair G53–C61 and the T-loop architecture, could be replaced respectively by the inverted base pair C53–G61 and by unusual anticodon loop or tetraloop sequences. Variants of whole $tRNA^{Asp}$ or of the 12 base-pair aspartate minihelix, with enlarged 13 base-pair long aminoacyl acceptor branches, as in selenocysteine-inserting tRNAs that are not recognized by elongation factor Tu, keep their binding ability to this factor. These functional results are well accounted for by the crystallographic structure of the *Thermus thermophilus* binary EF-Tu·GTP complex, which possesses a binding cleft accommodating the minimalist 10 base-pair domain of the tRNA aminoacyl acceptor branch.

In the cell, tRNAs[1] are involved in a large number of interactions with proteins. Some of the tRNA-specific proteins, such as aminoacyl-tRNA synthetases, select specifically the homologous tRNA, or a tRNA isoacceptor family, by recognizing a limited set of identity nucleotides shared only by these tRNAs (Giegé et al., 1993). On the contrary, activated EF-Tu·GTP has to recognize all aminoacylated elongator tRNAs present in the cell. Ternary complex formation of aminoacylated elongator tRNAs with EF-Tu·GTP is a crucial step for cell viability since formation of this complex permits the kinetically controlled entrance of aminoacyl-tRNA into the elongation cycle (Thompson et al., 1981). Since all tRNA sequences differ from each other, except for a set of conserved and semiconserved residues involved in the classical L-shaped folding (Steinberg et al., 1993), the activated EF-Tu has to recognize some characteristic structural features or well-conserved sequences carried by all tRNA species.

The process of recognition between tRNAs and translation factors is understood only in some limited cases. Thus, discrimination between $tRNA^{fMet}$ and elongator $tRNA^{Met}$ by bacterial EF-Tu is achieved by a missing 1–72 Watson–Crick base pair in the initiator tRNA (Seong & RajBhandary, 1987). The lack of $tRNA^{Sec}$ recognition by *Escherichia coli* EF-Tu·GTP was attributed to the unusual 13 base pairs in the aminoacyl acceptor branch (Baron & Böck, 1991). The canonical elongator tRNAs contain 12 base pairs in this region. In the initiator tRNA from plants and fungi, the discrimination from the elongation process is achieved by modification of the purine 64 residue (Kiesewetter et al., 1990). Footprinting (Boutorin et al., 1981; Wikman et al., 1982) and cross-linking (Wikman et al., 1987) experiments suggested that the T-loop, T-stem, aminoacyl stem, NCCA end, and variable loop of tRNA provide the major contact points with *E. coli* EF-Tu. Furthermore, Joshi et al. (1984) could demonstrate with a valylated tRNA-like domain derived from turnip yellow mosaic virus RNA that the shortest RNA fragment able to bind with the *E. coli* EF-Tu·GTP complex immobilized on a column contains the equivalent of the variable loop and the aminoacyl acceptor branch of tRNA. Despite these investigations, the molecular recognition between elongator aminoacyl-tRNAs and EF-Tu·GTP is not fully understood.

Following these lines, the goal in this work was to investigate the role of the tRNA-modified bases in ternary complex formation and to determine the size of the smallest aminoacyl-tRNA fragment able to interact efficiently with EF-Tu·GTP. The role of invariable features of tRNA, such as the conserved G53–C61 base pair at the end of the T-stem and the TΨC sequence in the T-loop, in ternary complex formation was also studied. Experiments were conducted with tRNA transcripts deprived of modified bases and with short RNAs recapitulating different parts of the aminoacyl acceptor region of tRNAs.

Yeast $tRNA^{Asp}$ has been chosen as the model tRNA for our studies since the X-ray structure of this tRNA is available (Moras et al., 1980; Westhof et al., 1985) as well as the solution structure of the unmodified transcript (Perret et al., 1990a). Further, it is known that this transcript shows no differences in aspartylation capacities when compared to the native yeast $tRNA^{Asp}$ (Perret et al., 1990b). Since identity residues conferring aspartylation specificity for this tRNA (Pütz et al., 1991) include residue 73 located in the aminoacyl acceptor branch, minimalist $tRNA^{Asp}$ versions still possessing aminoacylation capacity could be designed and used as substrates for elongation factor Tu from *Thermus thermophilus*. Indeed, this protein is able to interact with heterologous tRNAs including yeast tRNAs (Ott et al., 1989). Our results show that the main structural features in aminoacylated aspartate

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[1] Abbreviations: (AEDANS-s^2C_{75})Tyr-$tRNA^{Tyr}$, Tyr-$tRNA^{Tyr}$ modified at cytidine 75 with a thio group at position 2 of the pyrimidine ring to which an *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulfonic acid fluorescence group is attached; AspRS, aspartyl-tRNA synthetase; EF-Tu, elongation factor Tu; $EF\text{-}Tu_f$, nucleotide-free elongation factor Tu; tRNA, transfer ribonucleic acid.

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tRNA lie in the aminoacyl acceptor branch of the tRNA and do not involve the canonical T-loop structure. These data could be rationalized on the basis of the crystallographic structure of the binary complex EF-Tu·GppNHp (where GppNHp is a nonhydrolyzable GTP analogue) from thermophilic bacteria (Berchtold et al., 1993; Kjeldgaard et al., 1993).

MATERIALS AND METHODS

Materials. Yeast AspRS (Lorber et al., 1983) and T7 RNA polymerase (Wyatt et al., 1991) were prepared as described previously. EF-Tu·GDP and nucleotide-free EF-Tu ($EF\text{-}Tu_f$) from *T. thermophilus* HB8 were prepared as described by Limmer et al. (1992). $tRNA^{Asp}$ from yeast was purified by chromatography on BD-cellulose and Sepharose 4B columns after countercurrent distribution of bulk brewer's yeast tRNA (Boehringer-Mannheim, France SA, Meylan) (Dirheimer & Ebel, 1967; Romby et al., 1985). Oligonucleotides (18–58-mer) were synthesized on an Applied Biosystems 381A DNA synthesizer and purified either on a Nucleosil 120-5-C18 column (Bischoff Chromatography, Zymark-France, Paris) or on appropriate preparative polyacrylamide gels. L-[^{14}C]-Aspartic acid at 54 and 206 mCi/mmol was purchased from Amersham Buchler (Braunschweig, Germany). Restriction enzyme *Bst*NI was from New England Biolabs (Beverly, MA). Boehringer (Manheim, Germany) provided phosphoenolpyruvate, pyruvate kinase, T4 DNA ligase, polynucleotide kinase, and all ribonucleotides.

Synthesis of tRNA Transcripts. Plasmids containing yeast wild-type and mutant $tRNA^{Asp}$ synthetic genes were constructed according to Perret et al. (1990a). To allow transcription of these genes, base pair U1–A72 in the $tRNA^{Asp}$ sequence was replaced by G1–C72. Transcription of *Bst*NI-linearized genes was performed during 3 h at 37 °C in 40 mM Tris-HCl, pH 8.1, 22 mM $MgCl_2$, 1 mM spermidine, 5 mM dithioerythritol, 0.01% Triton X-100, 4 mM each of ribonucleoside triphosphate, 16 mM GMP, and the appropriate amount of T7 RNA polymerase. After phenol extraction, transcripts were ethanol precipitated and purified by denaturing 12% PAGE. Full-length transcripts were eluted from the gel by electroelution (Schleicher and Schuell, Dassel, Germany).

Synthesis of Minihelices and Helix–Loop Structures. Minihelices and helix–loop structures derived from yeast $tRNA^{Asp}$ were obtained by *in vitro* transcription of single-stranded templates. The templates were obtained by annealing an 18-mer oligonucleotide (minus strand of the T7 RNA polymerase promotor) to another oligonucleotide, complementary to the 18-mer, followed by the complement of the RNA sequence desired. For this purpose, equal molar concentrations of the two oligonucleotides were mixed in 0.3 M Tris-HCl, pH 8.1, and 0.15 M $MgCl_2$, incubated during 3 min at 65 °C, and then slowly cooled to room temperature. Transcription was performed during 3 h at 37 °C in 40 mM Tris-HCl, pH 8.1, 22 mM $MgCl_2$, 1 mM spermidine, 5 mM dithioerythritol, 0.01% Triton X-100, 4 mM each of ribonucleoside triphosphate, 5 mM GMP, and the appropriate amount of T7 RNA polymerase. The transcripts of minimalist RNAs were purified to single nucleotide resolution as described for full-length tRNA transcripts.

Aminoacylation Reaction. Aspartylation of minihelices and helix–loop structures was conducted at 20 °C whereas full-length tRNAs were aminoacylated at 37 °C. The reactions were performed in 25 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM ATP, bovine serum albumin at 0.1 mg/mL, 0.1 mM L-[^{14}C]aspartic acid, and the appropriate amount of yeast AspRS. All reactions were stopped by phenol extraction in the presence of sodium acetate, pH 4.5. Transcripts were ethanol precipitated, washed, dried, and resuspended in 10 mM sodium acetate, pH 4.5, in order to prevent spontaneous deacylation. Aspartylation levels were determined by 5% trichloroacetic acid precipitation on 3 MM Whatman filters.

Hydrolysis Protection Assays. These experiments were conducted as described previously by Pingoud and Urbanke (1980). They permit monitoring, in a nonquantitative manner, the ternary complex formation between the aminoacylated RNA, GTP, and EF-Tu since EF-Tu·GTP when bound to aminoacyl-tRNA protects the labile aminoacyl ester bound from spontaneous base-catalyzed hydrolysis. For this aim, *T. thermophilus* $EF\text{-}Tu_f$ was first dialyzed against a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 10 mM $MgCl_2$ and activated by incubation of $EF\text{-}Tu_f$ (40 μM) with GTP (400 μM) in 100 μL during 10 min on ice. The protection assays for each aspartylated RNA were then performed by incubation of aspartylated transcripts (30 pmol, taking into account the aspartylation level of each transcript) freshly resuspended in the above buffer with EF-Tu·GTP (300 pmol) in a final volume of 100 μL. Comparative assays were performed in parallel, with the activated protein replaced by buffer. In order to form the ternary complexes, the samples were kept at 4 °C for 5 min. To start the hydrolysis, the temperature was raised to 37 °C. Aliquots of 20 μL were removed at 0, 30, 60, and 90 min and spotted on 3 MM Whatman filters. After trichloroacetic acid precipitation, residual radioactivity was determined by liquid scintillation counting.

K_D Measurements. Equilibrium dissociation constants, K_D, of aspartylated transcripts for their interaction with *T. thermophilus* EF-Tu·GTP were determined by fluorescence titration assays, as described earlier in detail, using (AEDANS-s^2C_{75})Tyr-$tRNA^{Tyr}$ as a fluorescent reporter ligand (Ott et al., 1989) and the aspartylated RNA as a competitor. These experiments permit determination of the dissociation constants of the ternary complexes at equilibrium. The K_D for each set of mutants was determined with the same preparation of fluorescent reporter, and all experiments were repeated three to four times.

RESULTS

Interaction of Native and Unmodified Asp-$tRNA^{Asp}$ with Elongator Factor Tu·GTP. The sequence of yeast $tRNA^{Asp}$ with highlighted modified bases is given in Figure 1A. The corresponding transcript (Figure 1B) is obtained by *in vitro* transcription and differs from the native tRNA by the complete lack of modifications and the presence of a G1–C72 base pair at the top of the acceptor stem instead of the U1–A72 wild-type base pair which hinders efficient transcription. Both molecules could be aminoacylated up to 100% by the cognate yeast AspRS and present similar kinetic parameters for aspartylation (Perret et al., 1990b). The interaction of the two aminoacylated tRNAs with *T. thermophilus* EF-Tu·GTP was tested using two different methods. The hydrolysis protection assay (Pingoud & Urbanke, 1980) qualitatively demonstrates that aminoacylated native and unmodified $tRNA^{Asp}$'s are both protected from spontaneous hydrolysis of the ester bound in the presence of a 10-fold molar excess of EF-Tu·GTP, whereas the aspartyl residue is rapidly hydrolyzed from the tRNAs in the absence of EF-Tu·GTP (Figure 2A). The formation of ternary complexes is quantitatively measured by a fluorescence titration assay developed by Ott et al. (1989).

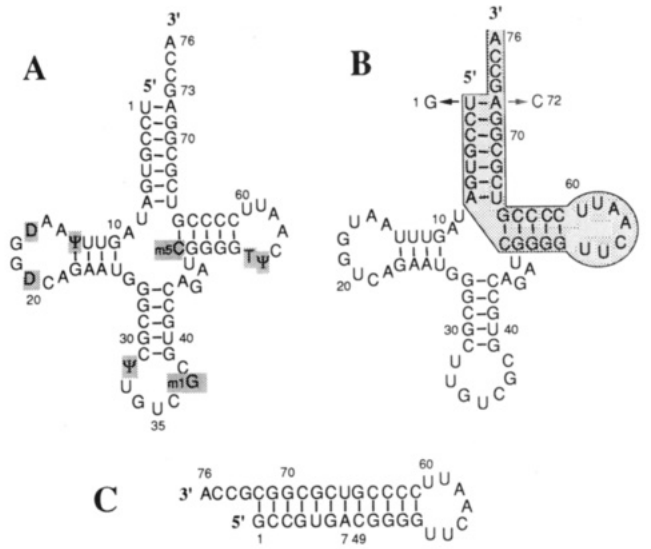

FIGURE 1: Cloverleaf structures of (A) wild-type yeast $tRNA^{Asp}$ emphasizing modified residues and (B) the yeast $tRNA^{Asp}$ transcript containing G1–C72 as the first base pair to allow efficient transcription and (C) sequence of the $minihelix^{Asp}$ derived from the aminoacyl acceptor branch of the $tRNA^{Asp}$ transcript as indicated by the shaded area in (B). Nucleotides are numbered according to Steinberg et al. (1993).

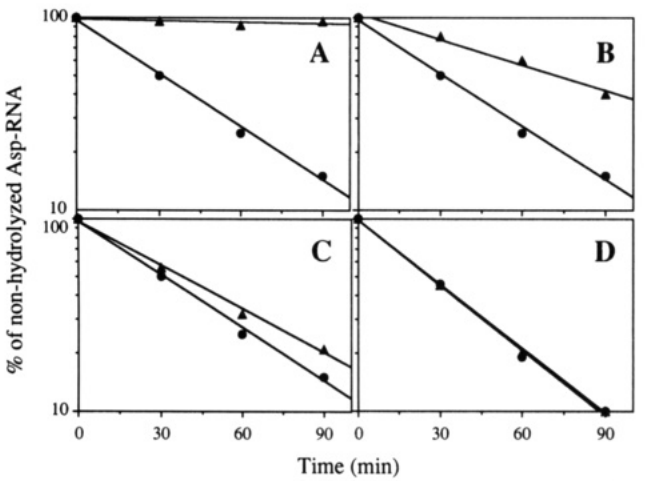

FIGURE 2: Rates of hydrolysis of the aspartate residue from different L-[^{14}C]Asp-RNAs in the presence (▲) and the absence (●) of EF-Tu·GTP. The concentration of Asp-RNA was 0.3 μM, and the concentration of EF-Tu·GTP was 3 μM. Panels: (A) aspartylated native $tRNA^{Asp}$ and the full-length $tRNA^{Asp}$ transcript, (B) aspartylated minihelices with 12 base pairs (see Figures 1C and 3a–c) (Asp-$tRNA^{Asp}$ with 13 base pairs in the aminoacyl acceptor branch and Asp–helix–loops with 13 and 11 base pairs provided very similar results and are depicted in this panel), (C) hydrolysis protection of the Asp–helix–loop with 10 base pairs, and (D) Asp–helix–loop structures containing 9, 8, and 7 base pairs, respectively.

In this experiment, Tyr-$tRNA^{Tyr}$ from yeast carrying a fluorescent reporter group at the penultimate nucleotide of the 3′-end is used as a ligand to detect the complex formation with EF-Tu·GTP. Aspartylated tRNA or RNA molecules compete with Tyr-$tRNA^{Tyr}$ and cause a reduction of the fluorescence signal. From titration of fluorescence-labeled Tyr-$tRNA^{Tyr}$ and Asp-RNA mixtures with EF-Tu·GTP, the equilibrium dissociation constant, K_D, can be determined. The dissociation constants for the interaction of native and unmodified $tRNA^{Asp}$ with EF-Tu·GTP are 1.6 and 2.6 nM, respectively (Table 1). Thus, native $tRNA^{Asp}$ binds only 1.6 times better to activated EF-Tu than does the unmodified $tRNA^{Asp}$ transcript.

Table 1: Equilibrium Dissociation Constants (K_D) of RNAs Charged with Aspartic Acid Measured by Fluorescence Titration According to Ott et al. (1989)

RNAs	K_D (nM)	L^a rel to unmodified $tRNA^{Asp}$ (x-fold)	L^a rel to $minihelix^{Asp}$ (x-fold)
aminoacylated tRNAs			
native yeast $tRNA^{Asp}$	1.6	0.6	0.25
unmodified $tRNA^{Asp}$	2.6	1	0.4
$tRNA^{Asp}$, 13 bp	1.8	0.7	0.3
aminoacylated RNA helices			
helix–loop, 13 bp	9.2	3.5	1.5
$minihelix^{Asp}$ (wt)	6.2	2.4	1
$minihelix^{Asp}$ (variant a)	9.1	3.5	1.5
$minihelix^{Asp}$ (variant b)	6.9	2.6	1.1
$minihelix^{Asp}$ (variant c)	8.1	3.1	1.3
helix–loop, 11 bp	8.6	3.3	1.4
helix–loop, 10 bp	27	10.4	4.3
helix–loop, 9 bp	126	48	20
helix–loop, 8 bp	190	73	31
helix–loop, 7 bp	268	103	43
uncharged RNAs			
tRNAs[b]	>10000	>3846	>1613
$minihelix^{Asp}$	>5130	>1973	>827

[a] Loss of affinity (L) is defined as the ratio $K_{D(mutant)}/K_{D(transcript^{Asp}\ or\ minihelix^{Asp})}$. L-values for repeated experiments varied by less than 25%. Minihelix refers to the standard length of 12 bp formed in canonical tRNA. bp = base pairs. [b] Data taken from Ott and Sprinzl (1992).

Interaction of Asp–Minihelix^{Asp} with Elongation Factor Tu·GTP. Since modified bases of tRNAs are not essential for ternary complex formation in the yeast aspartic acid system, all further experiments were performed with molecules prepared by *in vitro* transcription assays. A so-called minihelix (Figure 1C), corresponding only to the aminoacyl acceptor branch of the yeast $tRNA^{Asp}$ transcript, was enzymatically synthesized by *in vitro* transcription. This short molecule consisting of 35 nucleotides is aminoacylatable up to 30% by yeast AspRS (Frugier et al., 1944). EF-Tu·GTP causes a significant protection from deacylation of the aspartylated minihelix (Figure 2B). The affinity of EF-Tu·GTP for the minihelix as determined in equilibrium titration is 2.4-fold lower than for the complete $tRNA^{Asp}$ transcript (Table 1). Since aminoacylation of minihelices occurred only to 30%, experiments were performed with a population of aminoacylated and nonaminoacylated molecules. Although uncharged minihelices represent 70% of the whole population, their interaction with EF-Tu·GTP is at least 3 orders of magnitude lower than that of aminoacylated molecules (Table 1). Thus, uncharged molecules do not perturb significantly the interaction of the aminoacylated minihelices with EF-Tu·GTP.

In vitro transcription permits easy introduction of mutations into the RNA molecules. In a first series of experiments, variants of the aspartylatable minihelix, with an unchanged number of helical base pairs and selected mutations in the helix and the T-loop region, were tested. Three variants were created with altered conformation of the loop closing the minihelix (Figure 3). In variant a, the conserved base pair G53–C61 which stabilizes the canonical conformation of the T-loop (Quigley et al., 1975) was inverted to a C53–G61 base pair which does not permit H-bonding with phosphate 60 of the T-loop (Romby et al., 1987). In variant b, the 7-nucleotide-long anticodon–loop of $tRNA^{Asp}$ replaces the T-loop of the same length but of different conformation (Westhof et al., 1985). Finally in variant c, the T-loop was substituted by a 5′ C(UUCG)G 3′ sequence able to form a tetraloop with an

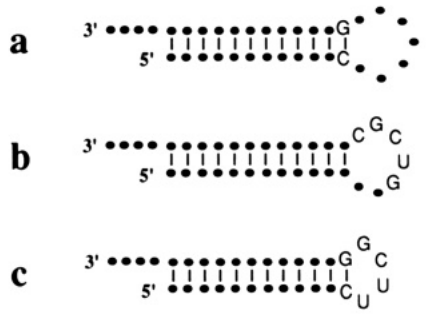

FIGURE 3: Schematic representation of mutated minihelices^{Asp}. Residues similar to the wild-type sequence (see Figure 1C) are indicated by black dots whereas those mutated are indicated by letters. Representations: (a) replacement of the invariant G53–C61 for C53–G61, (b) replacement of the T-loop for the anticodon–loop sequence, and (c) replacement of the T-loop by a tetraloop .

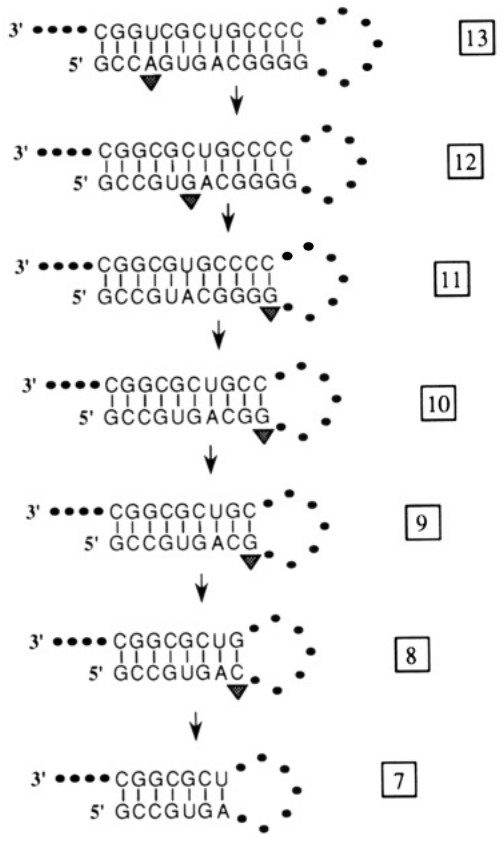

FIGURE 4: Sequences of helix–loops emphasizing the base pairs forming the aminoacyl acceptor branch. Black dots correspond to the wild-type, T-loop, and 3′-end sequences (see Figure 1C). The number of base pairs present in each helix is indicated in squares. Each helix–loop was derived from the preceding one by deletion of 1 base pair (indicated by triangles).

unusually high stability (Cheong et al., 1990). Aspartylation levels of approximatively 30% were reached with all three substrates. The interaction of EF-Tu·GTP with these aminoacylated minihelices was qualitatively demonstrated using hydrolysis protection assays (Figure 2B). From fluorescence titration assays, we determined that all three minihelix variants interact with EF-Tu·GTP with approximately the same K_D as that of the wild-type minihelix^{Asp} (Table 1).

The second set of mutants, displayed in Figure 4, presents a progressive reduction in the number of base pairs forming the aminoacyl acceptor branch. These molecules (helix–loops) present aminoacyl acceptor branches varying from 11 to 7 base pairs. A longer molecule containing 13 base pairs was also synthesized. All helix–loops could be aspartylated with plateau levels comprised between 20% and 30%. Typical hydrolysis protection assays for aspartylated helix–loops of 13 and 11 base pairs are shown in Figure 2B. The K_D values for these molecules are similar to that of the wild-type minihelix^{Asp} since they differ only by a factor of 1.5 and 1.4, respectively (Table 1). Hydrolysis protection assays performed with helix–loops containing 10 base pairs show a weaker protection from deacylation (Figure 2C), and helix–loops with 9, 8, and 7 base pairs show no protection at all (Figure 2D) as compared to the wild-type minihelix. These results are corroborated with the fluorescence titration experiments, since the resulting K_D values indicate a 4.3–43-fold lower affinity of these mutants for EF-Tu·GTP.

Interaction of Asp-tRNA^{Asp} Containing an Extended Aminoacyl Acceptor Branch with Elongation Factor Tu·GTP. A tRNA^{Asp} transcript presenting an additional A–U base pair between the third and the fourth base pair in the aminoacyl acceptor stem (tRNA^{Asp}, 13 bp) was synthesized and tested in ternary complex formation. This mutant is aspartylated up to 100% and binds to EF-Tu·GTP with a K_D value of 1.8 nM, as determined by an equilibrium fluorescence titration experiment (Table 1). Hydrolysis protection assays also show a clear protection from deacylation of this aspartylated transcript in the presence of EF-Tu·GTP (Figure 2B).

DISCUSSION

Determination of the Dissociation Constants of Aminoacyl-tRNA·EF-Tu·GTP Complexes. There is a discrepancy between the dissociation constant values for the aminoacyl-tRNA·EF-Tu·GTP interaction when determined by equilibrium methods using fluorescence reporter groups and the nonequilibrium, aminoacyl hydrolysis protection assays. The K_D's for aminoacyl-tRNA interaction with EF-Tu·GTP as measured by fluorescence are in the range of 0.19–6.4 nM for *E. coli* EF-Tu·GTP and *E. coli* aminoacyl-tRNAs (Abrahamson et al., 1985; Ott et al., 1990). Similar K_D values were obtained by the EF-Tu·GTP protection assay, where the unbound aminoacyl-tRNA is degraded in the presence of a large amount of ribonuclease (Louie et al., 1984). The hydrolysis of the aminoacyl ester bound in aminoacyl-tRNA occurs under conditions described in Figure 2, with a half-life of about 30 min. Binding of aminoacyl-tRNA to EF-Tu·GTP inhibits the rate of this spontaneous hydrolysis. The molecular details of this protection are not known. Most probably the aminoacyl residue of the aminoacyl-tRNA is bound as an orthoester acid to the protein (Förster et al., 1994). The formation of this orthoester structure, involving the ester carbonyl group, prevents hydrolysis. The stability of the orthoester will therefore be dependent on the local conformation in the aminoacyl residue binding site. We can therefore imagine a situation in which the polynucleotide chain is bound to the protein but the hydrolysis protection is disturbed. In such a case, the K_D's determined by fluorescence or ribonuclease protection may remain unchanged whereas the protection from spontaneous hydrolysis will be affected. There is still another difficulty in using hydrolysis protection for determination of K_D's for aminoacyl-tRNA·EF-Tu·GTP complex formation. In the time range used for this assay (90 min) the intrinsic GTPase converts a considerable amount of GTP to GDP (Chinali et al., 1977). This reaction affects the equilibrium of ternary complex formation, which is probably the main reason why the K_D's determined by the hydrolysis protection assay (Pingoud & Urbanke, 1980) are usually 2 orders of magnitude higher than the K_D's determined under equilibrium conditions. The data in Figure 2 should therefore

be considered only as a qualitative demonstration of the interaction. Due to the complexity of this assay, no quantitative conclusions can be drawn from these experiments. The fluorescence titrations, on the other hand reflect in quantitative manner the ability of the tested aminoacyl-RNAs to compete with (AEDANS-s^2C_{75})Tyr-$tRNA^{Tyr}$ for the EF-Tu·GTP binding site.

Nonimportance of Modified Bases for Interaction with EF-Tu·GTP. Unmodified tRNA transcripts present a more flexible structure as compared to the fully modified tRNAs (Hall et al., 1989; Perret et al., 1990a). They are efficient substrates for most cognate aminoacyl-tRNA synthetases, and the posttranscriptional modifications have been found only in two cases to act as identity elements for recognition by the cognate enzyme (Muramatsu et al., 1988; Sylvers et al., 1993). In the yeast aspartic acid system, it was demonstrated that these modifications are not required for aspartylation since both native and unmodified $tRNA^{Asp}$ molecules present similar kinetic parameters (Perret et al., 1990b). Here we show that aspartylated yeast $tRNA^{Asp}$ and its corresponding unmodified transcript are efficient substrates for activated *T. thermophilus* elongation factor Tu with dissociation constants in the nanomolar range. Modifications increase the K_D value only by a factor of 1.6 and thus do not bring a major contribution for ternary complex formation. Recently, similar results were obtained with the *E. coli* $tRNA^{Phe}$ transcript complexed with the homologous EF-Tu·GTP (Harrington et al., 1993). These results are not unexpected since several tRNA-like structures present at the 3′-ends of viral genomic RNAs are naturally deprived of base modifications, and they interact efficiently with procaryotic elongation factors [reviewed in Mans et al. (1991) and Florentz and Giegé (1994)]. Lack of a clear effect caused by tRNA modifications on dissociation constants, however, does not mean that minor nucleotides in tRNA are not important for translational fidelity (Harrington et al., 1993).

EF-Tu·GTP Does Not Require the Anticodon Branch for Binding Aminoacylated tRNAs. Aspartylated $minihelix^{Asp}$ interacts with EF-Tu·GTP with a K_D value similar to that of the $tRNA^{Asp}$ transcript. This demonstrates directly that the aspartylated aminoacyl branch of $tRNA^{Asp}$ contains all the structural information for an efficient interaction with EF-Tu·GTP. It shows further that the structure of the anticodon branch of tRNA does not activate (or only very moderately considering the 2.4-fold difference in K_D between whole tRNA and $minihelix^{Asp}$) by an allosteric-like mechanism the conformation of the aminoacyl branch to make it an efficient substrate for elongation factor Tu.

EF-Tu·GTP Interacting with the tRNA Aminoacyl Acceptor Branch Is Insensitive to T-Loop Structure. Some consensus elements, such as the G53–C61 base pair at the end of the T-stem, the TΨC triplet, and nucleotide A58 in the T-loop, are located in the aminoacyl acceptor branch of tRNAs. These elements could thus be good candidates for specifying common functions of tRNAs, in particular for EF-Tu·GTP recognition. The T-loop is of particular interest because 4 of its 7 residues are conserved in all tRNAs and govern its conformation (Quigley et al., 1975; Romby et al., 1987). Furthermore, earlier investigations gave no clear answer concerning the requirement of the T-loop in EF-Tu·GTP binding. Indeed, whereas some footprinting experiments showed no protection of the T-loop against nuclease digestion in the presence of the protein (Boutorin et al., 1981; Wikmann et al., 1982), other studies identify residues of the T-loop as cross-linking sites with EF-Tu·GTP (Wikmann et al., 1987). However, these conclusions were derived from experiments using bulky enzymatic probes or large-size cross-linking reagents and thus could not map fine structural features in the complexed tRNA.

Here we show that minihelix variants, mutated at the G53–C61 base pair adjacent to the T-loop or presenting an altered T-loop structure, interact efficiently with EF-Tu·GTP. Our results indicate clearly that the sequence of the last base pair of the T-stem, as well as the T-loop sequence, is not determining the interaction of charged tRNA with EF-Tu·GTP. From this it follows that the T-loop is likely not in direct contact with the protein. The existence of tRNAs that miss the canonical sequence of the T-loop, e.g., bovine mitochondrial $tRNA^{Ser}$, further supports this view since this bizarre molecule forms a ternary complex with bacterial EF-Tu·GTP (Gebhardt-Singh & Sprinzl, 1986). In summary, the present results demonstrate the unimportance of the T-loop structure for recognition of charged tRNAs by EF-Tu·GTP.

Role of the Aminoacyl Acceptor Branch Length for Interaction with EF-Tu·GTP. Since elongation factor Tu seems not to require any conserved sequences of the aminoacyl acceptor domain of tRNAs for an efficient binding, it is reasonable to assume that the protein recognizes a sugar–phosphate backbone of the aminoacyl acceptor branch helix. All elongator tRNAs share a 12 base-pair continuous helix in the aminoacyl acceptor branch formed by the coaxial stacking of the 7 base-pair acceptor arm and the 5 base-pair T-arm. To determine the extent to which this constant helix length is important in ternary complex formation, RNAs with enlarged or shortened helical length were assayed for interaction with elongation factor.

E. coli $tRNA^{Sec}$ contains 13 base pairs in its aminoacyl-accepting branch formed by the stacking of the 8 base-pair long acceptor arm and the 5 base pairs of the T-arm. This unusual tRNA is recognized by its own translation factor SELB (Forchhammer et al., 1989). Baron and Böck (1991) showed that reduction of the length of the $tRNA^{Sec}$ aminoacyl acceptor branch from 13 to 12 base pairs prevents the interaction of the variant $tRNA^{Sec}$ with SELB but induces its binding with EF-Tu·GTP, suggesting that the length of the accepting branch is crucial for recognition by elongation factors. Thus it was of particular interest to determine whether $tRNA^{Asp}$ and $minihelix^{Asp}$ follow the same rules. Surprisingly, mutants of the $tRNA^{Asp}$ transcript and $minihelix^{Asp}$, containing an additional A–U base pair positioned between base pairs 3–70 and 4–69 in the aminoacyl acceptor stem, interact efficiently with EF-Tu·GTP. Since EF-Tu·GTP fails to recognize $tRNA^{Sec}$, but recognizes both $tRNA^{Asp}$ and a helix–loop with 13 base pairs, we propose that other peculiar structural features of $tRNA^{Sec}$ are preventing the recognition by EF-Tu·GTP. The orientation of the large variable loop and the unusual set of tertiary interactions of $tRNA^{Sec}$ (Baron et al., 1993) are proposed to fulfill this role. Thus, the absence of a large variable loop in the $tRNA^{Asp}$ or in the 13 base-pair long helix–loop permits the binding to activated EF-Tu. We suggest that the large variable loop of $tRNA^{Sec}$ serves as an antideterminant to prevent the recognition by EF-Tu·GTP. Since the number of base pairs forming the aminoacyl acceptor stem is related to the helix rotation and thus determines the position of the large variable loop of $tRNA^{Sec}$, it is possible to understand why a mutant of the *E. coli* $tRNA^{Sec}$ having only 12 base pairs in the aminoacyl acceptor branch is recognized by EF-Tu·GTP (Baron & Böck, 1991).

In a second series of experiments, base pairs were progressively removed from the wild-type $minihelix^{Asp}$ until a helix–

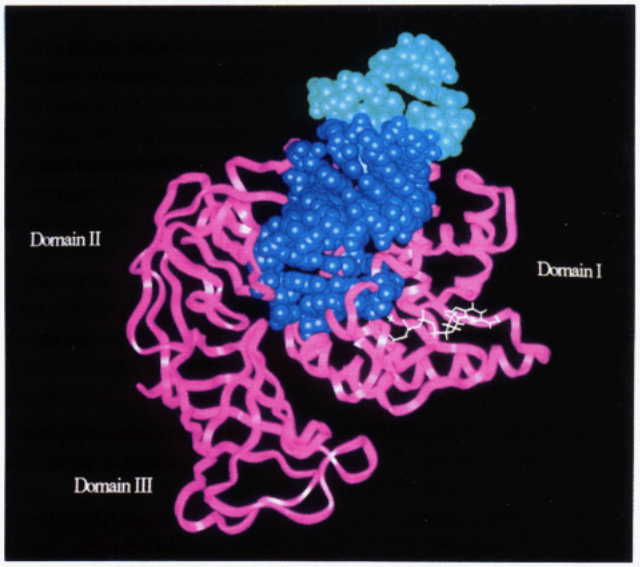

FIGURE 5: Model of the ternary complex formed between *T. thermophilus* EF-Tu, GppNHp, and the aminoacyl acceptor branch of yeast $tRNA^{Asp}$. The three domains of the protein appear in purple; Mg^{2+} and GppNHp are in white. $Minihelix^{Asp}$ is shown in different colors to emphasize the contacts with the protein: the first 10 base pairs that are in contact with EF-Tu are in dark blue. The last 2 base pairs of the T-stem and the T-loop are in green and are exposed to the solvent without participating in the interaction. Views were prepared using the Insight II system (Biosym software) and X-ray structure data of yeast $tRNA^{Asp}$ (Westhof et al., 1985) and *T. thermophilus* EF-Tu·GppNHp (Berchtold et al., 1993). In the construction of the model, van der Waals radii of the interacting molecules were taken into account.

loop structure of only 7 base pairs (length of the acceptor stem alone) remained. The location of the deleted base pair was not considered important, since there are no conserved sequences within the aminoacyl acceptor stems of tRNAs (Steinberg et al., 1993). The K_D values of these shortened aspartylated helix–loop structures for their interaction with EF-Tu·GTP are higher as compared to the K_D's of the 12 base pairs containing aspartylated $minihelix^{Asp}$ (Table 1). Whereas the helix–loops of 11 and 10 base pairs interact quite efficiently with EF-Tu·GTP (K_D's increased only by factors of 1.4 and 4.3, respectively, as compared to $minihelix^{Asp}$), those containing 9, 8, and 7 base pairs form much weaker complexes with the elongation factor Tu (K_D's increased by factors 20, 31, and 43, respectively, as compared to $minihelix^{Asp}$). This demonstrates that activated EF-Tu does not strictly require all 12 base pairs in the aminoacyl acceptor branch and shows that the minimal structure able to bind with highest affinity to EF-Tu·GTP, and thus containing all recognition elements necessary for binding to EF-Tu·GTP, is a helix–loop domain of 10 base pairs.

This ensemble of investigations suggests that *T. thermophilus* elongation factor recognizes an aminoacylated N_{73}-CCA 3′-end followed by a continuous helix of 10 base pairs. This recognition excludes the whole T-loop and the last 2 base pairs of the T-stem. In this context the importance of coaxial stacking of the aminoacyl stem and the T-stem of the tRNA for interaction with EF-Tu becomes obvious.

RNA Minihelix/EF-Tu·GTP Interaction Model and Evolutionary Considerations. Recently, the crystal structure of elongation factor Tu in its activated GTP state was elucidated at high resolution, suggesting a location of the binding site for aminoacyl-tRNAs (Berchtold et al., 1993; Kjeldgaard et al., 1993). As shown in Figure 5, *T. thermophilus* EF-Tu is a 44-kDa protein organized in three distinct domains. To form a ternary complex with aminoacyl-tRNAs, EF-Tu must be present in its active GTP-bound form. The comparison of the crystal structures of *E. coli* EF-Tu in its GDP state (Jurnak, 1985; Kjeldgaard & Nyborg, 1992) and of *T. thermophilus* EF-Tu in its GTP state emphasized the existence of large conformational changes in all three domains (Berchtold et al., 1993). One of the consequences of these rearrangements is the formation of a solvent-filled cleft between domains I and II of EF-Tu·GTP, exposing to the solvent several positively charged residues which thus become potential binding sites for the negative sugar–phosphate backbone of tRNAs. Earlier biochemical investigations performed on *E. coli* EF-Tu located the aminoacyl residue of aa-tRNA on domain I (Duffy et al., 1981; Jonák et al., 1982; Metz-Boutigue et al., 1989).

Since the crystal structures of elongation factor (Berchtold et al., 1993; Kjeldgaard et al., 1993) and of native yeast $tRNA^{Asp}$ (Moras et al., 1980; Westhof et al., 1985) are known, it becomes possible to propose a model of interaction between EF-Tu·GTP and $minihelix^{Asp}$. We deleted residues 8–48 of native yeast $tRNA^{Asp}$ as well as the single-stranded 3′-end, whose great flexibility does not allow prediction of its orientation in regard to the protein, and placed the remaining aminoacyl branch in the appropriate binding site of *T. thermophilus* EF-Tu by computer-guided molecular modeling. The model of interaction obtained is shown in Figure 5. Interestingly, it was not possible to integrate the entire aminoacyl acceptor branch of yeast $tRNA^{Asp}$ into the cleft of EF-Tu·GTP. Although the first 10 base pairs of the aminoacyl acceptor branch contact the protein, the last 2 base pairs are located outside domains I and II of the factor. This is in agreement with the abrupt increase of the K_D values measured for helix–loops containing less than 10 base pairs in the aminoacyl acceptor branch. Furthermore, the absence of contact between the entire T-loop and the protein explains that the K_D's are not influenced by replacing the T-loop by an anticodon–loop or a tetraloop.

Studies on tRNA aminoacylation systems using tRNA minihelices (Francklyn & Schimmel, 1989) and knowledge of the modular structures of aminoacyl-tRNA synthetases led to the tempting suggestion that contemporary synthetases and tRNAs could have evolved from simplified versions corresponding to RNA helices and the conserved catalytic cores of synthetases (Schimmel et al., 1993). The fact that elongation factors require only structural signals within the aminoacyl acceptor branch of tRNAs without any participation of the anticodon branch suggests that, in the primitive translation machinery, the elongation step has also emerged using the simplified versions of tRNAs chargeable by minimalist synthetases.

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