Structural Requirements of Escherichia coli Formylmethionyl Transfer Ribonucleic Acid for Ribosome Binding and Initiation of Protein Synthesis*

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R. MAYA SUNDARI, HEIKE PELKA, AND LADONNE H. SCHULMAN

From the Department of Developmental Biology and Cancer, Division of Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Escherichia coli formylmethionine tRNA can be isolated as two isomers which differ by a single nucleotide in the variable loop (mG versus adenosine). These two initiator tRNAs have been found to bind to ribosomes in an initiation factor and AUG-dependent reaction with identical kinetics. In addition, the bound tRNAs react with puromycin and initiate polypeptide synthesis at the same rates.

Conversion of the unusual 5'-terminal C3A5 base pair at the end of the acceptor stem of tRNA^met to a normal U3A2 base pair has previously been shown to greatly enhance the ability of this tRNA to interact with bacterial elongation factor, EF-Tu. We have now determined that this structural change has no detectable effect on the initiator properties of tRNA^met. In contrast to these results, fMet-tRNA^met containing a U-C-A sequence in place of the normal 3'-C-C-A terminus shows greatly reduced initiation factor-dependent ribosome-binding activity. In addition, most, if not all, of the bound tRNA is unable to undergo peptide bond formation with puromycin. Removal of the 5'-terminal phosphate from fMet-tRNA^met also reduces the stability of the ribosomal complex formed with the initiator tRNA. The data indicate the presence of two functionally distinct binding sites for 5'-hydroxy terminal fMet-tRNA^met which are not interconvertible under the conditions of the binding assay. One of these sites is equivalent to the usual P-site since the bound tRNA reacts with puromycin at the same rate as unmodified fMet-tRNA^met; the remainder of the bound tRNA is unable to participate in peptide bond formation.

Recent work from this laboratory has been concerned with determination of the structural requirements of Escherichia coli tRNAs for initiation and elongation of protein synthesis in bacteria (1-3). Recent studies on the recognition of E. coli formylmethionine tRNA by bacterial initiation factor IF-2 have indicated that in the absence of ribosomes, IF-2' cannot distinguish the nucleotide sequence of tRNA^met but recognizes the fMet moiety (1). In contrast, initiation factor-dependent ribosome binding of fMet-tRNA^met has an almost absolute requirement for the initiator tRNA sequence (4). In this communication, we report on the effects of structural alterations in tRNA^met on the ability of the tRNA to bind to the donor site on bacterial ribosomes and to initiate polypeptide chains in an in vitro protein-synthesizing system.

MATERIALS AND METHODS

\(^\text{14}C\)-labeled methionine (395 cpm/pmol) was purchased from New England Nuclear Corp. Poly(U,G) (3:1) was obtained from Miles Laboratories and puromycin dihydrochloride from Boehringer Mannheim. Lyophilized venom of Crotalus adamanteus was obtained from the Miami Serpentarium, Miami, Fla. Snake venom phosphodiesterase was purified from the crude venom by the procedure of Dolapchiev et al. (5). Escherichia coli tRNA^met purified from crude E. coli K-12 tRNA was as described previously (1). The procedures used for bisulfite modification of tRNA^met have been reported (6, 7). 5'-Terminal phosphate groups were removed from the tRNA by treatment with purified E. coli alkaline phosphatase at 65° as described earlier (2). The dephosphorylated tRNA contained no chain breaks and accepted the same amount of methionine and formate as untreated tRNA^met. The modified 3' terminus of bisulfite-treated tRNA^met was removed by digestion with purified snake venom phosphodiesterase and a normal 3'-terminal C-C-A sequence was reinserted using tRNA nucleotidyltransferase as described before (9). Assays for methionine and formate acceptor activities (8) and procedures for preparation of [\(^\text{14}C\)]Met-tRNA^met and [\(^\text{14}C\)]Met-tRNA^met have also been reported (9). Methods for the preparation of ribosomes, crude initiation factors, and high speed supernatant fraction (2) and the assay for initiation factor-stimulated ribosome binding of [\(^\text{14}C\)]Met-tRNA^met (1) as described earlier. Purified IF-1, IF-2, and IF-3 were kindly provided by Dr. Umadas Maitra of this department. Puromycin reactivity of ribosome-bound tRNA was assayed using the method of Leder and Bursztyn (10) except that incubations were at 25°. Puromycin was added after ribosome binding had reached plateau values for each tRNA. Polypeptide chain initiation was measured by poly(U,G)-directed incorporation of radioactivity from \(^\text{14}C\)-Met-tRNA^met into hot trichloroacetic acid-insoluble material using the procedure of Dubnoff and Maitra (11) except that the magnesium concentration was 10 mM and incubations were carried out at 37°.

RESULTS AND DISCUSSION

Escherichia coli formylmethionine tRNA (tRNA^met) is a mixture of two structures which differ by a single base at position 47 from the 5' terminus (12). The major isomer containing mG7 has been designated tRNA^met and the minor species containing A7, tRNA^met, have been reported (13, 14). The base change, which occurs in the variable loop of tRNA^met, alters the ter-
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Secondary structure of the tRNA (15-17), and affects the kinetic parameters for aminoaclylation by E. coli methionyl-tRNA synthetase (17, 18). We have now investigated the relative abilities of the two tRNAs to bind to E. coli ribosomes in an AUG and initiation factor-dependent reaction. Fig. 1 shows that there is no detectable difference in the rate or yield of binding. In addition, the tRNAs show the same kinetics of reaction with puromycin and rate of initiation of polypeptide chains in a poly(U,G)-dependent in vitro protein-synthesizing system (not shown). Thus the ordered structural differences between the two tRNAs cause no measurable difference in their ability to function as initiator tRNAs. The experiments described below were carried out with tRNA

Treatment of tRNA

with sodium bisulfite at 25°C results in conversion of 6 cytidine residues in the tRNA to uridine residues (6). Two of these modifications (C50 and C80) cause loss of amino acid acceptor activity while the remaining four modifications (C1, C1, C17, and C20) have no effect on aminoaclylation or subsequent formylation of the tRNA (7).

Bisulfite modification of tRNA

with 3 M sodium bisulfite at pH 6 for 18 h at 25°C resulted in the loss of about 90% of the original methionine acceptor activity. The remaining active molecules were modified to the extent of 95 to 100% at C1, 50 to 80% at C15, and 75 to 80% at C65. The modified tRNA was enzymatically aminoaclylated and formylated and tested for its initiation factor-dependent ribosome-binding activity in the presence of AUG (Fig. 2). The extent of binding with the modified tRNA was much less than that obtained for unmodified fMet-tRNA

In order to determine whether the C75 \rightarrow U65 base change affects binding, the modified tRNA was treated with snake venom phosphodiesterase under conditions leading to complete removal of the three hydroxy terminal nucleotides. A normal 3'-terminal C-C-A sequence was then resynthesized from CTP and ATP in the presence of tRNA nucleotidyltransferase. Fig. 2 shows that after repair of the damaged C-C-A sequence, the ribosome-binding activity of the bisulfite-modified fMet-tRNA

was substantially increased. These data indicate that the nucleotide sequence of the 3'-terminus of the initiator tRNA is important for formation of a stable complex with 70 S ribosomes in an initiation factor and AUG-dependent reaction.

The ability of fMet-tRNA

containing the modified 3'-terminal sequence to undergo peptide bond formation was investigated by measuring the puromycin reactivity of the ribosome-bound tRNA. Fig. 3 shows that about half of the bound tRNA is released by reaction with puromycin at the same rate as that observed with unmodified fMet tRNA

but the remainder is unreactive even after prolonged incubation with the drug. Since only about 75% of the bisulfite-treated tRNA has a modified CCA terminus and a substantial fraction failed to bind to ribosomes, it appeared likely that the

![Fig. 1. Initiation factor-stimulated ribosome binding of isomeric Escherichia coli initiator methionine tRNAs. Ribosome binding was carried out at 5 mM Mg2+ using AUG triplet as messenger as described before (1). f[14C]Met-tRNA

(20 pmol) plus initiation factors, ○—○; minus initiation factors, △—△; f[14C]Met-tRNA

(20 pmol) plus initiation factors, •—•; minus initiation factors, •—•; f[14C]Met-tRNA

(20 pmol) plus initiation factors, •—•; minus initiation factors, •—•.](http://www.jbc.org/)

![Fig. 2. Initiation factor-stimulated ribosome binding of bisulfite-modified fMet-tRNA

Ribosome binding was carried out as described in Fig. 1. f[14C]Met-tRNA

(16 pmol) plus initiation factors, ○—○; minus initiation factors, •—•; bisulfite-modified f[14C]Met-tRNA

(18 pmol) plus initiation factors, △—△; minus initiation factors, △—△; bisulfite-modified f[14C]Met-tRNA

having a repaired C-C-A sequence (17.5 pmol) plus initiation factors, •—•; minus initiation factors, •—•.](http://www.jbc.org/)

![Fig. 3. Puromycin reactivity of ribosome-bound fMet-tRNA

Puromycin reactivity was measured as described under "Materials and Methods." Results are expressed as per cent reaction of ribosome-bound fMet-tRNA

for each tRNA. f[14C]Met-tRNA

○—○; f[14C]Met-tRNA

△—△; f[14C]Met-tRNA

△—△; f[14C]Met-tRNA

having a repaired C-C-A sequence, •—•.](http://www.jbc.org/)
puromycin-reactive portion corresponded to those modified molecules containing a normal CCA terminus. This was confirmed by examining the puromycin reactivity of ribosome-bound bisulfite-modified fMet-tRNA\textsuperscript{Met} after repair of the damaged C-C-A sequence. The ability of the modified tRNA to react with puromycin was almost completely restored to the levels observed with unmodified fMet-tRNA\textsuperscript{Met} (Fig. 3).

Unmodified tRNA\textsuperscript{Met} has a non-hydrogen-bonded CCA sequence at the end of the acceptor stem. Bisulfite modification of the tRNA produces a C \rightarrow U base change which results in formation of a normal U-A-A base pair at the 5' terminus (6). This structural change has previously been shown to greatly increase the affinity of the initiator tRNA for elongation factor, EF-Tu, as measured by its ability to form an EF-Tu-GTP-aminoaeryl-tRNA ternary complex (2). The data shown in Figs. 2 and 3 using fMet-tRNA\textsuperscript{Met} having a fully base-paired acceptor stem indicate that this structural alteration has little or no effect on the ribosome binding and puromycin reactivity of the initiator tRNA. We also examined the ability of this modified fMet-tRNA\textsuperscript{Met} to initiate polypeptide chains using synthetic poly(U,G) as messenger RNA. This polymer efficiently stimulates initiation using the unmodified bacterial initiator tRNA which recognizes GUG as well as AUG as an initiator codon (11). The same rate of initiation was observed using fMet-tRNA\textsuperscript{Met} containing either a 5'-terminal cytidine or uridine (Fig. 4). Initial rates of polymerization varied with initiation factor concentration in the same manner for modified and unmodified Met-tRNA\textsuperscript{Met}. We also examined the ability of the unformylated tRNAs to initiate protein synthesis in order to determine whether the structural change at the 5' terminus resulted in any relaxation of the requirement for formylation in the bacterial system. A low level of initiation by unmodified Met-tRNA\textsuperscript{Met} is observed under the in vitro assay conditions, but both unformylated tRNAs initiated poorly compared to the corresponding formylated derivatives (Fig. 4). The formation of a base pair at the 5' terminus of the E. coli initiator tRNA therefore greatly enhances the ability of this tRNA to interact with bacterial elongation factor, EF-Tu, without altering its normal initiator function.

We have further investigated the structural requirements at the 5' terminus of fMet-tRNA\textsuperscript{Met} for ribosome binding by studying the binding properties of the initiator tRNA following removal of the 5'-terminal phosphate group. Fig. 5 shows that the dephosphorylated tRNA undergoes an initial rapid factor-dependent binding reaction but that binding plateaus at a substantially lower level than that observed with normal fMet-tRNA\textsuperscript{Met}. In addition, when the ribosome-bound tRNA was treated with puromycin, 70% reacted at a rate indistinguishable from that observed with Met-tRNA\textsuperscript{Met} containing a 5'-P group while the remainder failed to react even after prolonged incubation (not shown). These data indicate that the ribosome complex formed with fMet-tRNA\textsuperscript{Met} containing a 5'-OH group is less stable than that formed with the normal initiator tRNA and that only part of the dephosphorylated tRNA is bound in a way which permits subsequent peptide bond formation. Less difference between the two tRNAs was observed when rates of initiation of polypeptide chains were measured (Fig. 6). This was not unexpected since the partial reactions involved in protein synthesis have previously been found to reveal larger effects of tRNA structural alterations than are observed in overall in vitro polymerization reactions. For example, removal of the 5'-terminal phosphate from aminoaeryl-tRNAs drastically reduces the ability of these tRNAs to form a stable ternary complex with EF-Tu (2) but has less than a 2-fold effect on the rate of elongation of polypeptide chains in an in vitro protein-synthesizing system initiated by unmodified fMet-tRNA\textsuperscript{Met} (19). It is possible that significant rate differences would be observed for such structurally altered tRNAs in vivo under conditions of rapid cell growth.

Previous studies from other laboratories have shown that the C-C-A terminus plays a major role in the interaction of tRNAs with both the ribosomal donor and acceptor sites (20). Early investigations were carried out using substrate analogs containing 3'-terminal fragments of tRNAs which were found to act as peptide donors when alcohol was added to the reaction
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Fig. 6. Initiation of polypeptide synthesis by 5'-hydroxy terminal fMet-tRNA

REFERENCES

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