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Stability of the unique anticodon loop conformation of E.coli $tRNA_f^{Met}$

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ABSTRACT

Initiator tRNAs have an anticodon loop conformation distinct from that of elongation tRNAs as detected by susceptibility to S1 nuclease. We now find the anticodon loop conformation of *E.coli* $tRNA_f^{Met}$ to be stable under different salt conditions as detected by using S1 nuclease as a structural probe. In contrast, a conformational change is observed in the T- and D- loop of this tRNA in the absence of added Mg^{2+}. This change can be suppressed by spermine. Even under those conditions effecting a change in T- and D- loop conformation, the anticodon loop does not change. This suggests that the conformational shift is controlled by Mg^{2+} and restricted to the D- and T- loop region only without affecting the anticodon domain.

The use of S1 nuclease as a conformational probe requires the use of kinetic studies to determine the initial cleavage sites. Thus, the use of a strong inhibitor which immediately stops the action of this nuclease is necessary. ATP is shown to be such an inhibitor.

INTRODUCTION

In protein synthesis, the principal interaction between tRNA and the ribosome involves the tRNA anticodon triplet and the codon of mRNA. This interaction determines the sequence of amino acids found in the nascent polypeptide chain. We have previously found that the initiator tRNA anticodon loop conformation is different from that found in chain elongation tRNAs for both prokaryotic and eukaryotic systems (1). This conformational difference was detected using S1 nuclease as a structural probe. In chain elongation tRNAs, the S1 nuclease initially cleaves at least the two bases on the 3'-side of the anticodon triplet (base 35, 36) and in addition cleaves the constant U33 and base 34. In contrast, initiator tRNAs show initial enzymatic accessibility only to the central and 5' nucleotide of the anticodon. Our studies have found these differences consistently and they may be function-

ally significant within the ribosome (1) see also ref. 11.

In the present study we determine the stability of the unique anticodon loop conformation of *E.coli* $tRNA^{Met}_f$ (1). We examine the S1 nuclease accessibility of this loop as the pH and salt concentration are varied. Further, the dependence of tRNA conformation on the presence of Mg^{2+} and/or spermine is determined.

In order to use nucleases as structural probes, it is necessary to identify initial cleavage sites since these are the only true reflections of the conformation of the intact tRNA. These cleavage sites can be identified through nuclease kinetic studies. Such studies are possible only if there are rapid and strong inhibitors of the nucleases. We show that for S1 nuclease from *Aspergillus oryzae*, ATP is a strong inhibitor.

MATERIALS AND METHODS

Materials: *E.coli* $tRNA^{Met}_f$ was a generous gift of Dr. B.A. Roe. S1 nuclease from *Aspergillus oryzae* which has been purified from contaminating T1-nuclease was a generous gift by J. Vournakis. Commercial S1 nuclease from Boehringer Mannheim was also used. *Physarum I* nuclease was kindly provided by Dr. H. Donis-Keller. T1 nuclease (*Aspergillus oryzae*) (E.C.3.1.4.8), pancreatic nuclease A (E.C.3.1.4.22), calf intestine alkaline phosphatase (E.C.3.1.3.1) and polynucleotide kinase (E.C.3.1.4.22) were purchased from Boehringer Mannheim. [γ-^{32}P] ATP (3000 Ci/mmole) was purchased from New England Nuclear Company. The sterile Millex-Millipore filters are from Millipore Company, Bedford, Massachusetts.

Methods: This structure determining method is a modification of the rapid RNA-gel sequencing method (2-4) except that the single strand specific S1 endonuclease has been used under different salt conditions (4).

Preparation of [5'-^{32}P]-labeled *E.coli* $tRNA^{Met}_f$ –

0.1 A_{260} of *E.coli* $tRNA^{Met}_f$ was dephosphorylated with 0.2 units of calf intestine alkaline phosphatase in 25 mM Tris HCl, pH 8.0, at 55°C for 30 mins. in a 10 µl reaction volume. The reaction was stopped with 2 mM trinitrolotriacetic acid (NTA) for 20 mins. at room temperature to chelate the Zn^{2+} ions. It was then heat-

ed to 100°C for 1 min., chilled on ice and adjusted to 50 mM NaOAc, pH 5.0, and precipitated with 3 volumes of EtOH at -70°C for 30 mins. (3,4, 5).

100 pmole of dephosphorylated *E.coli* $tRNA_f^{Met}$ was adjusted to 25 mM Tris HCl, pH 8.0, 10 mM $MgCl_2$, 6 mM mercaptoethanol, 50 µg/ml bovine serum albumin, 5% glycerol and incubated with 4 units of polynucleotide kinase, 150 p mole of [γ-^{32}P]-ATP (3000 Ci/mmole) at 37°C for 1 hour (5) in a 20 µl reaction volume. To the reaction mixture 20 µl of 50 mM NaOAc, pH 4.5, 2 mM EDTA 8 M urea, 0.05% Bromphenol blue, 0.05% xylene cyanol was added and directly loaded to the gel.

The reaction mixture was purified on a 20% acrylamide gel in 7 M urea, 90 mM Tris borate, pH 8.3, 1 mM EDTA. The dimensions of the well are 3 cm x 0.3 cm (4) while the migration distance is 18 cm.

The tRNA was electrophoresed at 300 V for 15 hours until the xylene cyanol marker reached the bottom of the gel. The tRNA was identified by autoradiography for 2 or 5 minutes. The intact band was cut out, minced and eluted in 1 ml of 0.5 M NH_4Ac, 0.1% SDS, 10 mM $MgCl_2$ for four hours at room temperature. This elution mixture was passed through a syringe attached to a sterile Millex-Millipore filter (4). 10 A_{260} of unfractionated *E.coli* tRNA was added to the [5'-^{32}P]-labeled tRNA as a carrier for EtOH precipitation. The EtOH precipitation was repeated in order to remove traces of SDS and urea. After each precipitation, the pellet was dried down and redissolved in 250 µl of double-distilled bycovin-treated water. The tRNA was stored at 4°C (4).

Sequencing and Structural Analysis

In order to analyze correctly the S1 nuclease digestion patterns, a sequence determination was performed. The numbering of *E.coli* $tRNA_f^{Met}$ is according to Gauss *et al.* (6), where the three anticodon bases are C34, A35 and U36. Three sequence specific nucleases were used: T1 nuclease (cleaves after guanosine), pancreatic nuclease A (cleaves after pyrimidines and under partial digestion conditions it cleaves preferentially on the 5'-side of adenosine) and *Physarum I* nuclease (cleaves dominantly after uridines and adenosines, weakly after guanosines, but not

after cytosines). The reaction mix contained 25 mM NaOAc, pH 4.5, 7 M urea, 10 mM EDTA and it was incubated at 55°C for 30 minutes. The following enzyme:substrate ratios were used;

T1 nuclease:	0.05 - 0.005 units/1 µg tRNA
Pancreatic A nuclease:	0.004 - units/1 µg tRNA
Physarum I nuclease:	the units/µg in the preparation were unknown, so the appropriate amount was found by titration.

In determining the positions of the nuclease cleavage sites, incubation of tRNA in 50 mM NaOH, 1 mM EDTA at 100°C for 20 seconds gave a partial hydrolysis after each nucleotide. 2'-O-methylriboses were resistant to this treatment (3,4) which resulted in a gap in the electrophoresis ladder. For the structural investigation, S1 nuclease was used under different salt conditions as described in the text and figure legends.

RESULTS

Effects of pH and Salt Concentrations

[5'-^{32}P]-labeled *E.coli* $tRNA^{Met}_f$ was partially digested by S1 nuclease under two different reaction conditions: (a) 25 mM NaOAc, pH 4.5, 5 mM $MgCl_2$, 50 mM KCl. These are conditions under which aminoacylated tRNA is stable. (b) 40 mM cacodylate (KCac) (pH 6.0), 10 mM $MgCl_2$, 4 mM spermine tetrachloride, 25 mM $(NH_4)_2SO_4$. These are conditions under which orthorhombic crystals of *E.coli* $tRNA^{Met}_f$ are formed (7).

Partial hydrolysis of [5'-^{32}P]-labeled *E.coli* $tRNA^{Met}_f$ with S1 endonuclease under both conditions revealed two cleavage sites (Figures 1 and 2). These two bands do not line up with the bands generated by alkaline hydrolysis. Instead, they migrate such that the smaller fragment lies between C34 and A35. This reflects anomalous migration of the differing hydrolysis sites of S1 nuclease and alkali: S1 nuclease generates fragments ending for a 3'-OH group, while alkaline hydrolysis generates fragments ending with a 3'-phosphate. The identification of bands on the alkaline hydrolysis ladder was aided by the sequencing lanes, 2 - 6. In order to identify unambiguously the S1 nuclease cleavage sites in *E.coli* $tRNA^{Met}_f$, RNase A was used under nondenaturing conditions. RNAse A cleavage sites are seen in lanes 13

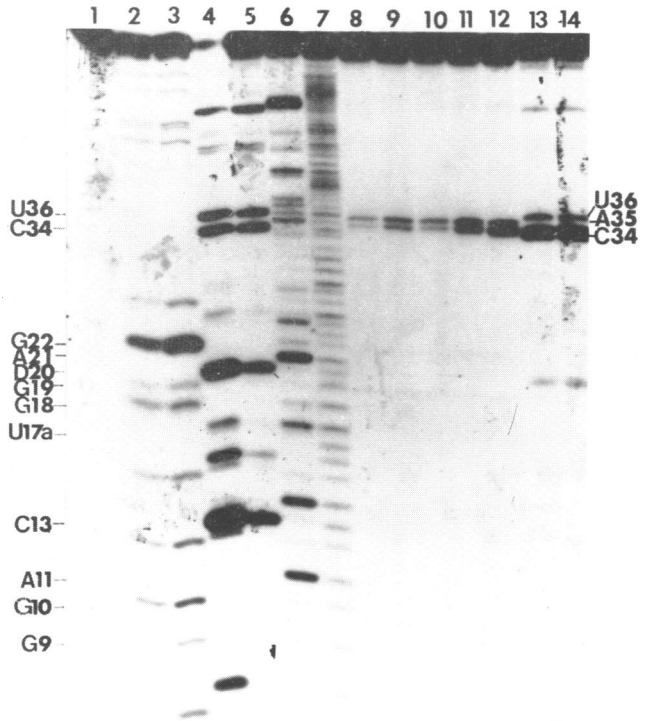

Figure 1: Autoradiogram of *E.coli* [5'-^{32}P] $tRNA^{Met}_{f}$.
Lane 1: no nuclease added.
Buffer system: 80 mM Kcac (pH 6.0), 10 mM $MgCl_2$, 50 mM NH_4SO_4, 8 mM spermine (2 x crystallization buffer).
Lanes 2-6 were in: 25 mM NaOAc (pH 4.5), 7 M urea, 1 mM EDTA, 60°C for 30 minutes.
Lanes 2 & 3: T1 nuclease, 0.5 units/µg tRNA and 0.05 units/µg tRNA.
Lanes 4 & 5: Pancreatic RNase A. 0.04 units/µg tRNA and 0.004 units/µg tRNA.
Lane 6: *Physarum I* nuclease.
Lane 7: 50 mM NaOH, 1 mM EDTA, 100°C for 20 seconds.
All reactions in Lanes 8 - 14 were preincubated without nuclease at 37°C for 10 minutes and incubated together with the nuclease for 20 minutes at 37°C.
Lane 8: S1 nuclease, 0.06 units/µg tRNA in 2 x crystallization buffer.
Lane 9: as in Lane 8, but 0.12 units/µg tRNA.
Lane 10: S1 nuclease, 0.06 units/µg tRNA in 1 x crystallization buffer.
Lane 11: as in Lane 10, but 0.12 units/µg tRNA.
Lane 12: S1 nuclease, 0.06 units/µg tRNA in 25 mM NaOAc (pH 4.5), 5 mM $MgCl_2$, 50 mM KCl.
Lane 13,14: Pancreatic RNase A, 0.04 units/µg tRNA in 1x resp. 2x crystallization buffer.

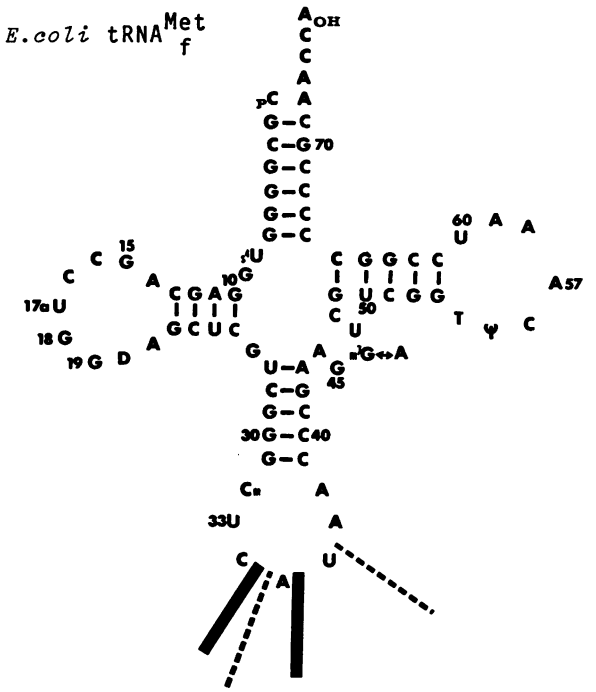

Figure 2: Cloverleaf structure of *E.coli* $tRNA_f^{Met}$ (9) showing the positions of S1 nuclease cleavage sites (solid line) and of RNAse A (dashed lines) under crystallizing conditions as described in the text and in the legend of Figure 1. The numbering system is according to Gauss *et al.*(6).

and 14 which show two intense bands corresponding to C34 and U36, both of which are pyrimidines located on the 5'-side of an adenosine, A35 and A37, respectively (8). The S1 nuclease cleavage fragments (lanes 8 - 12) migrate between these two RNAse A fragments. The upper larger S1 fragment in lane 12 migrates faster than the fragment ending with U36 of lane 13, while the lower smaller fragment in lane 12 migrates a little more slowly than the fragment in lane 13 ending with C34. S1 nuclease fragments migrate more slowly than RNAse A fragments because of the absence of a terminal 3' phosphate group. Therefore, the larger S1 fragment can end either with A35 or C34, but not with U36, while

the smaller one can only end with C34 because it is above the RNAse A fragment ending with C34 (lane 13). Since the two S1 nuclease fragments differ by only one residue in length, the larger fragment in lane 12 must end with A35 as shown diagrammatically in Figure 2.

In lanes 10 and 11 half the concentration of the crystallization buffer is used. In lane 11 the amount of S1 nuclease is also doubled. In both lanes only two cleavage sites are observed at C34 and A35. When the S1 nuclease digestion was performed under the standard salt conditions the same two cleavage sites were found (lane 12). S1 digestions were also carried out at pH 7.2 and 7.4 and they produced the same two cleavage sites (data not shown). These results show that the cleavage pattern is stable under varying ionic conditions, at different pHs and at different S1 nuclease concentrations.

Effects of Mg^{2+} and Spermine

The stability of the anticodon loop conformation was also examined under conditions in which the Mg^{2+} concentration was varied from 0 -10 mM, using 25 mM NaOAc at pH 4.5 as buffer. These results are shown in Figure 3. In the absence of added Mg^{2+} (lane 7, Figure 3), major cleavage sites appear in the D loop and stem at A21 and G22, in the T loop at C56 or A57 (the precise identification remains to be made), as well as in the anticodon loop at C34 and A35 as before. A number of other, smaller fragments can also be seen which are probably secondary cleavage products. If 5 to 10 mM Mg^{2+} are added to the reaction mix, only the major cleavage sites in the anticodon loop are seen (lanes 8, 9, Figure 3). The bands, in addition to C34 and A35 seen in lanes 8 and 9, are probably due to contaminating T1 nuclease.

In the absence of added Mg^{2+}, 10 mM spermine also suppressed all S1 nuclease cleavage sites except those in the anticodon loop (lane 13, Figure 3). The presence of both Mg^{2+} and spermine in the reaction mix does not affect the cleavage pattern; i.e., there is no difference between the cleavage patterns when only Mg^{2+} or spermine are present, or when both are present.

In lane 10, 1 mM ATP was added to the reaction mix. This had the effect of reducing the radioactivity of the two anti-

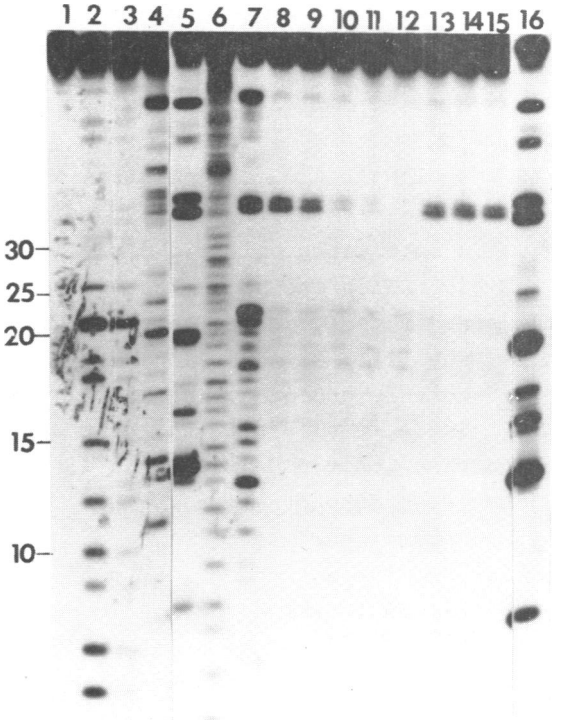

Figure 3: Autoradiogram of *E.coli* [5'-^{32}P] $tRNA_f^{Met}$, showing the effect of Mg^{2+}, spermine and ATP on S1 nuclease digestions.
Lane 1: no enzyme added.
Buffer system: 25 mM NaOAc (pH 4.5), 5 mM $MgCl_2$, 50 mM KCl. All reactions in Lanes 2 - 5 were performed in 25 mM NaOAc (pH 4.5), 7 M urea, 1 mM EDTA, 62°C for 30 minutes.
Lane 2: T1 nuclease, 0.5 units/µg tRNA.
Lane 3: Same as Lane 2, but 0.05 units/µg tRNA.
Lane 4: *Physarum I* nuclease.
Lane 5: Pancreatic ribonuclease A, 0.004 units/µg tRNA.
Lane 6: 50 mM NaOH, 1 mM EDTA, 100°C for 20 seconds.
Lane 7: S1 nuclease, 0.5 units/µg commercial S1 nuclease in 25 mM NaOAc (pH 4.5), 0 mM $MgCl_2$.
Lane 8: Same as Lane 7, but 5 mM $MgCl_2$.
Lane 9: Same as Lane 7, but 10 mM $MgCl_2$.
Lane 10: S1 nuclease, 0.5 units/mg in 25 mM NaOAc, 1 mM ATP.
Lane 11: Same as Lane 10, but 2 mM ATP.
Lane 12: Same as Lane 10, but 5 mM ATP.
Lane 13: S1 nuclease, 0.5 units/µg tRNA in 25 mM NaOAc, (pH 4.5), 0 mM $MgCl_2$, 10 mM spermine.
Lane 14: Same as Lane 13, but 5 mM $MgCl_2$.
Lane 15: Same as Lane 13, but 10 mM $MgCl_2$.
Lane 16: Pancreatic RNase A, 0.004 units/µg tRNA, 25 mM NaOAc (pH 4.5), 5 mM Mg^{2+}, 50 mM KCl.

codon loop bands by 90% in comparison to lane 8. Thus, ATP is a strong inhibitor of S1 nuclease even at low concentration. In lane 12, the addition of 5 mM ATP is seen to completely inhibit S1 nuclease. However, the continued presence of bands corresponding to G18, G19 and G22 in lanes 10 - 12 and particularly the absence of an effect of ATP on their intensity, reinforces the interpretation that these cleavage fragments are products of contaminating T1 nuclease in the commercial S1 nuclease. These T1 nuclease cleavage sites are dominant when a structural investigation using T1 nuclease is carried out under the above conditions (data not shown).

DISCUSSION

Our previous finding that initiator tRNAs exhibit an anticodon loop conformation distinct from that seen in elongation tRNAs (1) has led us to examine the stability of that conformation. One earlier result showed that initiator tRNAs were initially cleaved by S1 nuclease at C34 and A35 only. In contrast, elongation tRNAs are cleaved at positions 33, 34, 35 and 36 (1, 3, 4).

The results described here show that in *E.coli* $tRNA^{Met}_{f}$ the anticodon loop conformation is stable under a variety of conditions. In particular, the anticodon loop conformation is stable even under conditions that disrupt the conformation of other regions of the molecule. In the absence of Mg^{2+}, the D and T loops unfold so that both become accessible to S1 nuclease digestion. Spermine prevents this unfolding but has no detectable effect on the anticodon conformation of the anticodon loop. A comparison of *E.coli* $tRNA^{Met}_{f}$ with two elongation tRNAs, yeast $tRNA^{Phe}$ and *E.coli* $tRNA^{Glu}_{2}$, revealed that the degree of conformational changes due to Mg^{2+} and spermine in the D- and T- loop domain differs between these three tRNAs while the anticodon domain of these three tRNAs remained unaffected (4). In the case of yeast $tRNA^{Phe}$ the change in Mg^{2+} and spermine concentration showed minor changes in the D-loop part only. *E.coli* $tRNA^{Glu}_{2}$ could change its D-loop conformation to a larger extent when the physical environment was changed although the D- and T- loop did not separate as in *E.coli* $tRNA^{Met}_{f}$ (4). Whether this drastic confor-

mational change in *E.coli* $tRNA^{Met}_{f}$ is specific for initiator tRNAs is not clear yet.

Detection of the first S1 nuclease cleavage site provides information about exposed single-stranded regions of tRNAs. Kinetic studies have to be performed to determine the first cleavage position. No change in the anticodon loop cleavage pattern was found in the time interval 30 seconds to 30 minutes of *E.coli* $tRNA^{Met}_{f}$ (1). Normally, the appearance of additional bands during a kinetic study indicates secondary cleavage sites. Secondary cleavages occur mainly in the immediate vicinity of a primary site, usually one or two nucleotides away (4). In elongation tRNAs, like yeast $tRNA^{Phe}$, the cleavage after U33 occurs shortly after the cleavages at position 36 and position 35 (4, J. Vournakis, personal communication).

On the basis of the identity of S1 cleavage patterns in eukaryotic and prokaryotic initiator tRNAs, we have suggested that the conformation of the anticodon loop may be governed by the base-sequence of the adjoining anticodon stem (1). The lack of an effect of separation and cleavage of the T- and D- loops on the anticodon loop cleavage pattern would appear to support this hypothesis. The stability in the conformation of the anticodon loop, in contrast to that of the T- and D- loop, supports the suggestion that conformational differences in the anticodon loops of initiator versus elongation tRNAs may be functionally significant (1,10).

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