Role of Anticodon Bases in Aminoacylation of Escherichia coli Methionine Transfer RNAs*

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Previous studies from this laboratory have shown that a cytidine to uridine base change at the wobble position of the anticodon of Escherichia coli formylmethionine tRNA (C35) or at one of the cytidine residues of the 3'-terminal C-C-A sequence (C47) results in loss of methionine acceptor activity. In this paper, we show that inactive tRNA\textsuperscript{Met} containing the C35 \rightarrow U35 base change inhibits aminoacylation of active tRNA\textsuperscript{Met} significantly when present in an approximately equimolar concentration. In contrast, the C47 \rightarrow U47 base change not only eliminates methionine acceptor activity, but also prevents effective binding of tRNA\textsuperscript{Met} and methionyl-tRNA synthetase. Inactive tRNA\textsuperscript{Met} containing uridine at position 35 is unable to inhibit aminoacylation of active tRNA\textsuperscript{Met} even when present in a 30-fold molar excess. These results indicate that C35 plays an essential role in the recognition of tRNA\textsuperscript{Met} by its aminoacyl-tRNA synthetase.

The noninitiator methionine tRNA of E. coli contains the minor base N4'-acetylcytidine in the wobble position of the anticodon (ac\textsubscript{C35}). In order to determine whether the N4'-acetyl group affects enzyme recognition, we have developed a method for converting ac\textsubscript{C35} in tRNA\textsuperscript{Met} to an unmodified cytidine residue. Comparison of the kinetics of aminoacylation of tRNA\textsuperscript{Met} containing ac\textsubscript{C35} with that of tRNA\textsuperscript{Met} containing C35 has shown that removal of the acetyl group from the anticodon base has no effect on either the rate or the yield of methionine acceptance by this tRNA.

The structural differences between cytidine, uridine, and N4'-acetylcytidine are confined to the N1 and C4 positions of the pyrimidine ring. The results presented here indicate that the exocyclic amino group at C4 is not involved in aminoacylation and that E. coli methionyl-tRNA synthetase is not sensitive to small structural changes at this site. We propose that the enzyme interacts with the N3 position of the anticodon wobble cytidine of tRNA\textsuperscript{Met} and tRNA\textsubscript{ac}\textsuperscript{Met}.

Previous work from this laboratory (1-6) has been concerned with determination of the structural requirements for aminoacylation of Escherichia coli methionyl tRNAs by E. coli methionyl-tRNA synthetase. In earlier studies, we reported that a cytidine to uridine base change in the wobble base of the anticodon of tRNA\textsuperscript{Met} results in loss of methionine acceptor activity (3). In the present paper, we report additional studies on the effects of structural alterations of this nucleotide on recognition of both tRNA\textsuperscript{Met} and tRNA\textsubscript{ac}\textsuperscript{Met} by E. coli methionyl-tRNA synthetase. These studies indicate that the enzyme interacts with a specific functional group of the wobble base.

EXPERIMENTAL PROCEDURES

Materials

Sodium metabisulfite was Sigma Grade I reagent. [\textsuperscript{14}C]Methionine was purchased from New England Nuclear Corp. Aminex A-6 and Aminex A-25 were obtained from Bio-Rad Laboratories. Ribothymine was purchased from Sigma. N4'-Acetylcycltidine was prepared by the method of Otter and Fox (7). Poly(C) was prepared from poly(C) using the method of Michelson and Grunberg-Manago (8). This procedure consistently yielded polyribonucleotides having a molecular content of 35 to 38% N4'-acetylcytidine and 62 to 68% cytidine. RNase \textsubscript{T1} was obtained from Calbiochem and bacterial alkaline phosphatase from Worthington Biochemicals. Snake venom phosphodiesterase was purified from the crude venom of Crotalus adamanteus by the procedure of Dolapev et al. (9).

Crude Escherichia coli K12 tRNA was obtained from General Biochemicals and used for the preparation of E. coli tRNA\textsuperscript{Met} having a specific activity of 1600 pmol/A\textsubscript{260} unit as described previously (5). E. coli tRNA\textsuperscript{Met} having a specific activity of 900 pmol/A\textsubscript{260} unit was isolated by the same chromatographic procedures. The tRNA contained 0.8 \pm 0.2 mol of ac\textsubscript{C}mol of methionine accepted. E. coli methionyl-tRNA synthetase was purified from E. coli K12 strain EM20031 carrying the F32 episome as described by Cassio and Waller (10). The enzyme had a specific activity of 9 units/mg in the aminoacylation assay of Lemoine et al. (11). Partially purified methionyl-tRNA synthetase was prepared as described before (6).

Methods

Bisulfite Modification of RNA – A solution containing 2.2 M NaHSO\textsubscript{3}, 0.11 M Tris, and 0.011 M MgCl\textsubscript{2} was adjusted to pH 7.5 with 10 N NaOH. RNA was added and the volume was adjusted such that the reaction mixture contained 10 A\textsubscript{260} units/ml of RNA, 2.0 M  

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N4-Acetylcytidine - RNA samples containing alkali-labile N4-acetyl- 
previously (3). Control samples of RNA were taken through the 
carried out at 37°. Bisulfite was removed by successive dialysis 
by incubation in 0.1
acceptor activity (3, 17). Separation of partially modified 
sites in the tRNA and is accompanied by loss of methionine 
residues to uridine residues (14-16). Previous studies from this 
laboratory have shown that treatment of 
stranded regions, resulting in conversion of these cytidine 
analysed on Aminex A-25 was performed at 25°. Samples that did not 
Aminex A-25 at the same position. Flow rates were maintained at 20 
using the procedure of Uziel 
equipped with a Sargeant-Welsch recorder.

-1 uridine/mol of RNA, and 80 units/ml of T7 ribonuclease. After incubating 
for 16 h at 37°, 50 µl of 0.2 M Tris/formate, pH 7.8, and 0.8 unit of 
bacterial alkaline phosphatase were added and incubation was con-
tinued for 12/2 min at 37°. The reaction mixture was then chilled and the 
ph readjusted to 4.5 by the addition of 20 µl of 1.0 M sodium 
formate, pH 4.0. Samples of tRNA were digested in the same way, 
but dilute buffers (0.01 M sodium formate, pH 4.5; 0.02 M Tris/
formate, pH 7.8; and 0.1 M sodium formate, pH 4.0) were used 
in order to minimize interference from impurities in the buffers 
that absorb in the ultraviolet and chromatograph near N*-acetylcytidine 
on Aminex A-25.

Nucleoside mixtures were analyzed on columns (0.5 x 30 cm) of 
Aminex A-25 by elution with 10 mM sodium formate, pH 4.0. When RNA samples contained ribothymine as well as N*-acetycytidine, 
analysis on Aminex A-25 was performed at 25°. Samples that did not 
contain ribothymine were chromatographed at 40°, since the time 
required for analysis is shortened considerably at the higher temper-
ature. At 40°, ribothymine and N*-acetycytidine are eluted from 
Aminex A-25 at the same position. Flow rates were maintained at 20 
to 36 ml/h using a Milton Roy minipump, generating pressures of 200 
and the flow rate was kept below 30 ml/h, in order to facilitate separation 
of ribothymine from N*-acetylcytidine. The column effluent was 
monitored at 254 nm through an 8-ml recording cell (Altex model 153) 
equipped with a Sargent-Welsch recorder.

Nucleoside analysis of major bases was carried out on a Aminex A-6 
using the procedure of Uziel et al. (12).

Aminoacylation Assays - Methionine acceptor activity was mea-
sured under the standard assay conditions described previously (6). 
Initial rates of aminoacylation of RNA,50 samples (Fig. 5) were 
determined by incubating the tRNA for 4 min at 25° in the reaction 
mixture described before (6) except containing 15.6 µM (14C)methio-
nine (200 to 500 µCi/µmol) and only 1 to 3 µg of protein enriched in 
methtionyl-tRNA synthetase. Other kinetic experiments were car-
ried out as described earlier (2) using purified methionyl-tRNA 
synthetase. Under both sets of conditions, aminoacylation was linear 
with time and proportional to enzyme concentration.

Enzymatic Recovery of the 3' Terminal of Bisulfite-modified 
tRNA50 - The modified 3' terminal nucleotides of bisulfite-treated 
tRNA50 were removed by limited digestion with purified snake 
venom phosphodiesterase and a normal 3'-terminal C C A sequence 
was synthesized using E. coli tRNA nucleotidyltransferase as de-
scribed before (13).

RESULTS

Effect of C → U Base Changes in Escherichia coli tRNA50 on the Ability of the tRNA to Interact with E. coli Methionyl-
tRNA Synthetase - It is known from the work of others that 
treatment of polynucleotides with sodium bisulfite at pH 6.0 
leads to deamination of cytidine residues located in single-
stranded regions, resulting in conversion of these cytidine 
residues to uridine residues (14-16). Previous studies from this 
laboratory have shown that treatment of E. coli tRNA50 under these conditions results in C → U conversions at six sites in the tRNA and is accompanied by loss of methionine 
acceptor activity (3, 17). Separation of partially modified 
tRNA50 into molecules which are biologically active and 
inactive has shown that base changes at C3, C16, C35, and C37 
do not prevent aminoacylation, while C → U conversions at either C35 or C37 inactive the tRNA (3). The active fraction, 
isolated by the phenoxacytidination procedure (18), contains 
an average of about 0.4 uridine/modified site and can be amino-
acylated to the extent of 90% of unmodified tRNA50.

We compared the kinetics of aminoacylation of active bisul-
finite-modified tRNA50 and unmodified tRNA50 (data not 
shown). The close similarity between the two sets of data 
dicated that the modifications present in the active bisulfite-
treated tRNA have little effect on the interaction of tRNA50 
with E. coli methionyl-tRNA synthetase. Examination of the 
kinetics of aminoacylation of active modified tRNA50 in the 
presence of an approximately equal amount of inactive modi-
fied tRNA50 showed a 2-fold reduction in the rate of methion-
yny acceptance, indicating that inactive molecules inhibit 
aminoacylation. Since the inactive modified tRNA used in this 
experiment was modified to the extent of about 0.5 uridinomeq of 
tRNA at each of the sites C35 and C37, most of the molecules 
in the mixture were modified at only one of these two sites. In 
order to determine whether tRNA50 modified at both sites 
inhibits aminoacylation, the kinetics of aminoacylation of active 
bisulfite-modified tRNA50 were examined in the pres-
ence of heavily modified inactive tRNA50 in which essentially 
all reactive cytidine residues had been converted to uridine. 
No inhibition was observed when the ratio of inactiveactive 
tRNA50 was varied from 1:1 to 60:1, indicating that tRNA50 containing both U35 and U37 does not bind effectively to E. coli 
methtionyl-tRNA synthetase.

In order to examine separately the effects of the C35 → U35 
and the C37 → U37 base changes, we removed the 3'-terminal 
U35-U36-A7 sequence from the bisulfite-treated tRNA by exo-
nucleolytic cleavage with snake venom phosphodiesterase and 
synthesized a normal C35-C36-A7 sequence using RNA nucleo-
cliditransferase. The resulting inactive modified tRNA containing 
~1 uridine/mol of tRNA at position 35 was tested for its 
ability to inhibit aminoacylation. No detectable inhibition 
was observed at ratios up to 30:1, inactiveactive tRNA50. 
Thus, inactive molecules containing the C35 → U35 base 
change are unable to bind effectively to the enzyme while 
inactive molecules containing the C37 → U37 base change bind 
readily, inhibiting aminoacylation.

Procedure for the Conversion of N*-Acetycytidine to Cytidine - The importance of C35 in aminoacylation of E. coli 
tRNA50 prompted us to investigate the effect of a naturally 
occurring structural alteration found at this site in the nonini-
tiator methionine accepting species, tRNA50. This tRNA has an 
N*-acetycytidine residue in the wobble position of the 
tandem and is the only tRNA in E. coli containing this 
minor base (19). In order to determine the effect of the acetyl 
group on the interaction of tRNA50 with E. coli methionyl-
tRNA synthetase, we developed a method whereby acetyl 
residues could be quantitatively converted to the parent unmodified 
cytidine.

Treatment of a model polynucleotide, poly(C35-C37), with 
sodium bisulfite at pH 7.5 was found to result in a decrease in 
the mC4 content of the polymer, accompanied by a commensur-
ate increase in the cytidine content (Fig. 1). The sum of mC4 and 
C, relative to an internal adenosine marker, remained 
-2 We use the term "effectively" to indicate an interaction between 
tRNA and E. coli methionyl-tRNA synthetase which either leads to 
product or inhibits product formation with active tRNA.
Fig. 1. Bisulfite-catalyzed conversion of N4-acetylcytidine to cytidine at pH 7.5. Poly(C2,ac4C) was incubated at a concentration of 880 nmol of nucleotide/ml in the presence of 2.0 M NaHSO3, pH 7.5, at 37°C, as described under "Experimental Procedures." The reaction mixture also contained poly(A), at a concentration of 143 nmol of nucleotide/ml, as an internal standard. The base composition of the T2 ribonuclease and alkaline phosphatase hydrolysates of aliquots removed at various times was determined on Aminex A-25 at 40°C. O—O, N4-acetylcytidine; ●●●●, cytidine; X—X, the sum of N4-acetylcytidine + cytidine, relative to adenosine.

Conversion of N4-acetylcytidine to cytidine by treatment with mild base has been reported previously (20). We have observed that the free nucleoside ac4C undergoes quantitative conversion to cytidine in both neutral and mildly alkaline solutions, with a half-life of about 4 h at 37°C, pH 9. In contrast, incubation of poly(C2,ac4C) under the same conditions for 9 h results in less than 5% loss of ac4C from the polymer, emphasizing the dramatic difference in stability of the minor base when present in polynucleotides. Kruppa and Zachau (21) have reported the conversion of N4-acetylcytidine in yeast tRNA1Met to unmodified cytidine by incubation at 37°C in concentrated ammonia. These harsh reaction conditions lead to substantial breakdown of the tRNA, however, in contrast to the procedure described here which results in quantitative deacetylation of ac4C with no concomitant phosphodiester bond cleavage.

Conversion of N4-acetylcytidine to uridine—Incubation of poly(C2,ac4C) with sodium bisulfite at pH 6.0 (deaminating conditions) caused a much more rapid loss of ac4C from the polymer than had been observed at pH 7.5 (Fig. 2). During the initial phase of the reaction, the amount of cytidine was found to rise above the starting level. This increase in cytidine was quantitatively equivalent to the decrease in N4-acetylcytidine. Thus, the major fate of N4-acetylcytidine under these conditions is rapid conversion, first to its bisulfite adduct and, then, to the bisulfite adduct of cytidine. The cytidine adduct, in turn, is converted to the bisulfite adduct of uridine, but more slowly. Thus, incubation of poly(C2,ac4C) under these conditions leads to deacetylation of N4-acetylcytidine, as well as to deamination of cytidine. The yield of uridine obtained following pH 9 treatment of the bisulfite-modified poly(C2,ac4C) was the same as that obtained from poly(C) which had been reacted in the same manner (not shown). It was not possible, however, to determine whether N4-acetylcytidine itself can undergo bisulfite-catalyzed deamination, or whether the bisul-
Acetylcytidine or Unmodified Cytidine in the Anticodon—

Conversion of N^4-acetylcytidine in tRNA^{Met} to Unmodified Cytidine— Sodium bisulfite treatment of tRNA^{Met} at pH 7.5 was carried out under conditions identical to those used for converting N^4-acetylcytidine in poly(C_aC) to cytidine. A control sample of tRNA^{Met} was incubated in a parallel reaction mixture containing 2.0 M NaSO_4, pH 7.5, instead of 2.0 M NaHSO_3, pH 7.5, but otherwise the same. After 24 h of incubation at 37°C, both samples were digested with T2 ribonuclease and alkaline phosphatase. The nucleoside mixture was chromatographed on Aminex A-25 at 25°C. Adenosine and guanosine, which elute after N^4-acetylcytidine, are not shown.

Fig. 3. Nucleoside analysis profiles of tRNA^{Met} treated with sodium bisulfite and with sodium sulfate. The tRNA^{Met} was treated with either 2.0 M NaSO_4 (a) or with 2.0 M NaHSO_3 (b), for 24 h at pH 7.5 and 37°C, as described under “Experimental Procedures.” Samples were digested with T2 ribonuclease and alkaline phosphatase. The nucleoside mixture was chromatographed on Aminex A-25 at 25°C. Adenosine and guanosine, which elute after N^4-acetylcytidine, are not shown.

Kinetics of Aminoacylation of tRNA^{Met} Containing N^4-Acetylcytidine or Unmodified Cytidine in the Anticodon—

When the methionine acceptor activity of tRNA^{Met} in which N^4-acetylcytidine had been converted to cytidine using sodium bisulfite was measured in the presence of excess *E. coli* methionyl-tRNA synthetase it was found that the bisulfite-treated tRNA retained more than 95% of the amino acid acceptor activity of the untreated tRNA (Fig. 4). In contrast, treatment of tRNA^{Met} with bisulfite under deaminating conditions (pH 6.0) resulted in pseudo-first order loss of methionine acceptor activity, at a rate identical with that observed for tRNA^{Met} incubated in a parallel experiment. These results underscore the fact that no deamination occurs under the conditions used for removal of the acetyl group from ac^3C in tRNA^{Met}, since deamination is accompanied by loss of amino acid acceptor activity.

In order to determine whether the absence of the acetyl group from N^4-acetylcytidine affected the interaction of tRNA^{Met} with *E. coli* methionyl-tRNA synthetase, the kinetic parameters for aminoacylation of tRNA^{Met} treated with 2.0 M NaHSO_3, pH 7.5, for 24 h were determined and compared with those obtained for untreated tRNA^{Met} (Fig. 5). These data show that the conversion of N^4-acetylcytidine in the anticodon of tRNA^{Met} to cytidine has no effect on the K_m or V_max for aminoacylation. In addition, the data in Fig. 5 show that the structural difference in the anticodon of tRNA^{Met} and tRNA^{Met} (ac^3C versus C_3C) does not account for the difference in K_m of the two isoacceptor tRNAs seen here and observed previously by others (22).

DISCUSSION

The data presented here show that a C → U base change in the anticodon of tRNA^{Met} not only prevents aminoacylation but also prevents effective binding of tRNA^{Met} to *E. coli* me-
thionyl-tRNA synthetase. This could arise from a conformational change in the tRNA resulting from the anticodon base change or could indicate an essential role for C35 in the recognition of tRNA^Met by E. coli methionyl-tRNA synthetase. The available information on the three-dimensional structure of tRNAs, including tRNA^Met, indicates that the anticodon loop is not involved in tertiary structure interactions and that the anticodon sequence is single-stranded (23, 24). In order to determine whether the experimental manipulations involved in bisulfite treatment of tRNA^Met introduce a conformational change in the molecule, we recently investigated the solution structure of the tRNA by high resolution NMR spectroscopy.

The 360 MHz NMR spectrum of unmodified tRNA^Met, containing approximately 25% secondary and tertiary N—H hydrogen bond resonances, was compared with that of bisulfite-modified tRNA^Met, containing six C → U base changes at positions 1, 16, 17, 35, 75, and 76. The modified tRNA, which had only 1.5% of the methionine acceptor activity of the unmodified tRNA, showed no loss of secondary or tertiary base pair resonances. Thus, the loss of biological activity of bisulfite-modified tRNA^Met and its lack of ability to bind effectively to the enzyme appear to reflect a requirement for a specific nucleotide in the anticodon of tRNA^Met for recognition by E. coli methionyl-tRNA synthetase.

The structural differences between cytidine, uridine, and N4-acetylcytidine are confined to the N3 and C4 positions of the pyrimidine ring. The requirement for a specific base at a particular site in the tRNA could reflect the requirement of the enzyme for interaction with a particular functional group or repulsion due to the presence of an incorrect functional group. The results presented here showing that removal of the acetyl group from acC35 in tRNA^Met has no effect on the kinetics of aminoaacetylation indicate that the presence or absence of a free exocyclic amino group is of no importance to the interaction of the tRNA with E. coli methionyl-tRNA synthetase and further indicate that the enzyme is not sensitive to small structural changes at this site, including the presence of a carboxyl group. We therefore favor the hypothesis that a positive interaction occurs between E. coli methionyl-tRNA synthetase and the N3 position of the anticodon base C35.

The observation that aminoaacetylation of certain tRNAs is drastically affected by single base changes has also been made in the case of E. coli tRNA^Thr (25-29), E. coli tRNA^Ala (30), E. coli tRNA^Glu (31), and yeast tRNA^Val (32). Yarus et al. (33) have suggested that the middle base of the anticodon of E. coli Su^+; tRNA is a ligand of E. coli methionyl-tRNA synthetase in the mischarging reaction which results from an anticodon base change in E. coli tRNA^Met.

The mechanism by which a base change in the anticodon of tRNA^Met can prevent recognition by E. coli methionyl-tRNA synthetase remains unclear. Nucleotide bases in regions outside of the anticodon have previously been shown to be involved in aminoaacetylation of this tRNA (4, 5) and the initial binding reaction probably involves additional structural elements which are not specific to tRNA^Met, since noncognate tRNAs have been shown to bind nonproductively to the enzyme (34). Interactions between other tRNAs and aminoaacetyl-tRNA synthetases are known to involve both general binding sites, which give stability to associations between any tRNA species and a particular enzyme, and unique binding sites which are responsible for the specific interaction between an enzyme and its cognate tRNA (55, 36). The loss of effective binding, leading to loss of aminoaacetylation activity and to loss of inhibitory capacity, which results from a single base change in the structure of tRNA^Met therefore indicates an important specific function for C35 in the aminoaacetylation mechanism.

A static model of aminoaacetylation, in which an initial binding step is followed immediately by a catalytic step appears insufficient to explain the role of C35. Since binding involves a number of interactions, a single nucleotide base would not be expected to provide a large differential increment to the binding specificity. The dramatic loss of ability of tRNA^Met to effectively interact with E. coli methionyl-tRNA synthetase following the C35 → U35 conversion suggests a more dynamic model of aminoaacetylation in which interaction of the enzyme with C35 and several other specific sites in tRNA^Met induces a structural rearrangement of the complex which is obligatory for correct positioning of the terminal adenine at the catalytic site. The lack of inhibition by tRNA^Met containing U35 suggests that the tRNA containing this base change is unable to undergo the conformational transition and is released rapidly from the enzyme surface. In contrast, the ability of tRNA^Met containing the C35 → U35 base change to inhibit aminoaacetylation suggests that “recognition” of this altered tRNA can still occur, and that the structural change near the 3′ end of tRNA^Met interferes with the catalytic step.

Other evidence indicates that native E. coli methionyl-tRNA synthetase may undergo conformational changes during aminoaacetylation. The dimeric enzyme has very similar or identical subunits (37) and has been shown to have two potentially equivalent tRNA^Met binding sites, but it exhibits anti-cooperative interaction with this substrate (38). This suggests that the initial binding of 1 molecule of tRNA^Met may promote a conformational change in the enzyme structure which prevents effective binding of the tRNA to the second site (38). Investigations of the binding of tRNA^Met in the presence of methionyl adenylate, the alkyl analog of methionyl adenylate, have shown that addition of either tRNA^Met or tRNA^Met to the E. coli methionyl-tRNA synthetase-methionyl adenylate complex is accompanied by a large change in the intrinsic protein fluorescence, indicative of a major conformational change of the enzyme (39).

Data supporting a dynamic model of tRNA-aminoaacetyl-tRNA synthetase recognition have recently been reported by a number of laboratories (33, 40-45).

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