Location of Accessible Bases in *Escherichia coli* Formylmethionine Transfer RNA as Determined by Chemical Modification†

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ABSTRACT: Chemical modification of *Escherichia coli* tRNA^{fMet} with 1 M chloroacetaldehyde, pH 5.5–6.0 at 25 °C, has been found to result in alteration of six cytidine and five adenosine residues in the molecule. The modified cytidine residues are the same as those previously found to be reactive with sodium bisulfite at pH 6.0. The accessible adenosine residues are A_{36} in the anticodon, A_{58} in the TΨC loop, and A_{73}, A_{74}, and A_{77} in the 3′ terminal sequence. No modification of adenosine residues in the dihydrouridine or variable loops or of adenosine residues on the 3′ side of the anticodon loop could be detected. Treatment of fMet-tRNA^{fMet} with chloracetaldehyde gave the same pattern of modification as was observed with deacylated tRNA^{fMet}. Chemical modification of *E. coli* tRNA^{fMet} with 2 sodium bisulfite, pH 7.0 at 25 °C, resulted in selective modification of exposed uridine residues in the tRNA. Only three sites were found to be reactive: U_{18} in the dihydrouridine loop, U_{37} in the anticodon, and U_{48} in the variable loop. The overall pattern of chemical modification of tRNA^{fMet} is very similar to that found by others for yeast tRNA^{Phe}, supporting the idea that many of the tertiary interactions in the two tRNAs are the same. The adenosine residue at position 58 in the center of the TΨC loop of the initiator tRNA shows unusual reactivity, however, being modified by chloroacetaldehyde at the same rate as the 3′ terminal adenosine residue. This result is in sharp contrast to the uniform resistance of nucleotides in the TΨC loop of yeast tRNA^{Phe} to chemical modification.

Chemical modification studies have been useful in probing the ordered structure of purified tRNA species in solution. The most detailed studies have been carried out on yeast tRNA^{Phe}, where specific chemical reagents have been used to determine the location of all of the accessible bases in the structure (Cramer et al., 1968; Igo-Kemenes and Zachau, 1969, 1971; Litt, 1971; Rhodes, 1975). The results of these studies have been compared with those expected on the basis of the known position of exposed bases in the three-dimensional crystal structure of yeast tRNA^{Phe} (Robertus et al., 1974a; Rich and Raj Bhandary, 1976). The excellent correlation between the location of chemically reactive bases and exposed bases has strongly supported the idea that the solution and crystal structures of this tRNA are essentially the same and has also provided strong evidence for the previous assumption that, under controlled experimental conditions, only exposed bases in tRNAs are susceptible to chemical attack. Nucleotides involved in duplex structure or in maintaining tertiary structure through stacking or base-pairing interactions are unreactive.

In this paper, we report on the location of exposed adenosine and uridine residues in *E. coli* tRNA^{fMet} [1] as measured by chemical modification. This work, together with previous solution studies on tRNA^{fMet} (Goddard and Schulman, 1972; Chang, 1973; Schulman et al., 1974), completes the determination of chemically accessible bases in a second class-I tRNA and allows for the comparison of the solution structure of an initiator with a noninitiator tRNA species.

Materials and Methods

Chloroacetaldehyde was purchased from Pfaltz and Bauer, diluted to the desired concentration with water, and distilled under reduced pressure. Sodium metabisulfite was Sigma Grade I reagent. Sodium [^{35}S]sulfite and [^{14}C]methionine were obtained from New England Nuclear Corp. Aminex A-6 and A-25 were purchased from Bio-Rad Laboratories. RPC-5 was obtained from Astro Enterprises, Inc. RPC-3 was prepared by the procedure of Kelmers et al. (1971).

E. coli tRNA^{fMet} was purified from crude *E. coli* K12 tRNA as described previously (Schulman, 1971). The two isomers of tRNA^{fMet} differing by an adenosine or 7-methylguanosine residue at position 47 from the 5′ terminus were separated by chromatography on RPC-3 as described by Weeren et al. (1972). fMet-tRNA^{fMet} was prepared as reported previously (Schulman and Her, 1973).

Lyophilized venom of *Crotalus adamanteus* was obtained from the Miami Serpentarium, Miami, Florida. Snake venom phosphodiesterase was purified from the crude venom by the procedure of Dolapchiev et al. (1974). Pancreatic RNase (RNase A) and RNase-free alkaline phosphatase were obtained from Worthington Biochemicals. RNase T_1 was purchased from Calbiochem and heat treated to destroy phosphatase activity (Schulman et al., 1974).

Chloroacetaldehyde-modified tRNA^{fMet} was digested with RNase T_1 as described previously (Schulman, 1971) and the resulting oligonucleotides were chromatographed on a 0.5 × 100 cm column of RPC-5 using a linear gradient of 0.11–3.2 M ammonium acetate, pH 7.4, over 800 ml at a flow rate of 36 ml/h. Three-milliliter fractions were collected and desalted by lyophilization. Pancreatic RNase digestions of chloroacetaldehyde-modified tRNA^{fMet} (40 A_{260}/ml) were carried out using 100 μg/ml of RNase A in 0.1 M Tris-HCl, pH 7.5, for 16 h at 37 °C, and the resulting oligonucleotides were chro-

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[1] Abbreviations used are: εA, 1,N^6-ethenoadenosine; εC, 3,N^4-ethenocytidine; tRNA$^{fMet}_1$, the *E. coli* initiator methionine tRNA having a m^{7}G residue at position 47 from the 5′ terminus; tRNA$^{fMet}_3$, the same tRNA having an adenosine residue at position 47; tRNA^{fMet}, a mixture of tRNA$^{fMet}_1$ and tRNA$^{fMet}_3$; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl.

matographed on RPC-5 as above using a linear gradient of 0.075–1.6 M ammonium acetate, pH 9.2, over 400 ml. Bisulfite-modified tRNA^{fMet} (12 A_{260}/ml) was digested with 450 units/ml of RNase T_1 in 0.1 M Tris-HCl, pH 7.0, for 2.5 h at 37 °C. The resulting oligonucleotides were chromatographed as described above, except that buffers were adjusted to pH 6.0 to prevent reversal of uridine-bisulfite adducts. Bisulfite-modified RNase T_1 oligonucleotides were further digested with 2.5 μg of RNase A/A_{260} of oligonucleotide in 25 μl of 0.1 M Tris-HCl, pH 7.0, for 2.5 h at 37 °C. The resulting products were chromatographed on RPC-5 as described above using a linear gradient of 0.11–1.0 M ammonium acetate, pH 6.0, over 400 ml. When ^{35}S-labeled sodium bisulfite was used for modification of tRNA^{fMet}, 3-ml fractions were collected and 0.5–1.0-ml aliquots were counted in 15 ml of Aquasol (New England Nuclear Corp.) in a liquid scintillation counter. Absorbance patterns were recorded at 260 nm with a Gilford Model 2400 absorbance recorder with a full scale of 0.1 to 3.0.

tRNA and oligonucleotides were enzymatically digested to nucleosides as described before (Goddard and Schulman, 1972). Samples were analyzed for U, G, A, C^m, εA, and m^7G on Aminex A-6 at pH 4.7 as described by Uziel et al. (1968). Under these conditions, εC comigrated with cytidine. Samples were analyzed for C, U, εC, and G on a 0.6 × 30 cm column of Aminex A-25. Elution was carried out using 10 mM ammonium formate, pH 3.62, at 50 °C and a flow rate of 30 ml/h. Under these conditions, εA and A were only partially resolved. Samples were analyzed for Ψ and rT on the same Aminex A-25 column run at room temperature. Nucleoside standards of purified εA and εC were kindly provided by Dr. Nelson Leonard.

5′ Terminal nucleoside analyses were carried out by digestion of 0.1–0.3 A_{260} of oligonucleotide in 25 μl of 0.02 M magnesium acetate, 0.2 M Tris-acetate, pH 8.8, with 4–8 units of purified snake venom phosphodiesterase for 30 min at room temperature. Duplicate reaction mixtures containing RNase-free alkaline phosphatase were used to analyze for total nucleoside composition of each sample. All modified oligonucleotides were found to be completely digested under these conditions. Nucleoside absorbance profiles were recorded at 254 nm using a Chromatronix Model 220 absorbance detector and a Sargent-Welch SRG Recorder with a full scale of 0.01–2.56.

Chloroacetaldehyde Modification of tRNA^{fMet}. Reaction mixtures contained 20 A_{260}/ml of tRNA^{fMet} and 0.5–1.0 M chloroacetaldehyde in 20 mM potassium acetate, 10 mM magnesium acetate, pH 5.0, 5.5, or 6.0. After reaction at 25 or 37 °C for various times, aliquots were removed and 1/10 volume of 1 M KCl, 0.5 M Tris-HCl, pH 7.5, and 2 volumes of ethanol were added. The tRNA was collected by centrifugation and reprecipitated twice from 0.1 M KCl, 10 mM $MgCl_2$, 0.05 M Tris-HCl, pH 7.5, with 2 volumes of ethanol. Fluorescence emission spectra of modified tRNA samples were recorded on an Amino-Bowman spectrophotofluorometer at room temperature.

f[^{14}C]Met-tRNA^{fMet} was modified with 1 M chloroacetaldehyde at pH 5.5 for 8 h at 25 °C as described above. After the reaction, an aliquot of the sample was checked for cold trichloroacetic acid-insoluble radioactivity and it was found that the tRNA had undergone 9% deacylation during the modification procedure. Another aliquot of the sample was hydrolyzed with base and analyzed by paper chromatography as described before (Sundari et al., 1976). The radioactivity migrated identically to that released by base hydrolysis of the starting f[^{14}C]Met-tRNA^{fMet}, indicating that no alteration of the fMet moiety had occurred during the chloroacetaldehyde treatment.

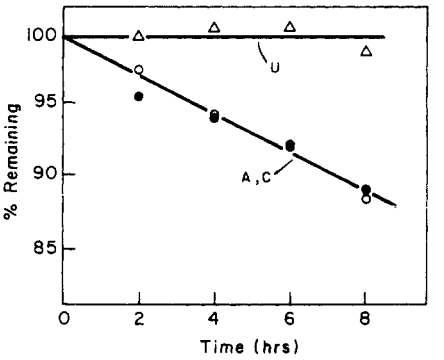


FIGURE 1: Chloroacetaldehyde modification of adenosine and cytidine residues in purified *E. coli* tRNA^{fMet}. Reaction mixtures contained 0.7 M chloroacetaldehyde in 10 mM magnesium acetate, 20 mM potassium acetate, pH 5.5. Incubation was at 25 °C. The nucleoside content of each sample was measured as described under Materials and Methods following enzymatic degradation of the modified tRNA. Results are expressed as the percentage change relative to guanosine. (○-○) A; (●-●) C; (△-△) U.

Bisulfite Modification of tRNA^{fMet}. Reaction mixtures contained 30 A_{260}/ml of tRNA^{fMet} in 10 mM $MgCl_2$, 2.0 M sodium bisulfite, pH 7.0. After reaction at 25 °C for various times, aliquots were removed, diluted with 2 volumes of water, dialyzed twice against 1000 volumes of 0.15 M NaCl, 20 mM sodium acetate, pH 4.5, once against 0.15 M NaCl, 20 mM sodium acetate, pH 6.0, and, finally, against 20 mM sodium acetate, pH 6.0. When desired, bisulfite adducts were completely reversed by incubation in 0.1 M Tris-HCl, pH 9, at 37 °C for 8 h.

Results

Properties of the Chloroacetaldehyde Reaction with tRNA. Chloroacetaldehyde is known to react with both adenosine and cytidine derivatives in weakly acidic aqueous solution to yield fluorescent products (Kochetkov et al., 1971; Barrio et al., 1972; Secrist et al., 1972). A substantial difference in the pH optimum for modification of cytidine (pH 3.5) and adenosine (pH 4.5) has previously been noted (Kochetkov et al., 1971), suggesting the possibility that specific modification of adenosine residues in tRNA might be achieved above pH 5. The rate of modification of adenosine and cytidine in crude *E. coli* tRNA was therefore examined as a function of pH. Adenosine residues were modified more rapidly than cytosine residues in crude tRNA; however, rates were independent of pH in the range 5.0–6.0. Thus, the large pH effects found with monomeric substrates were not observed with tRNAs. An increase in reaction temperature from 25 to 37 °C caused an increase in both the rate and extent of modification.

Chloroacetaldehyde-modifications of purified *E. coli* tRNA^{fMet} were carried out at pH 5.5 or 6.0 and at 25 °C.[2] Under these conditions, first-order loss of both adenosine and cytidine was observed, suggesting that different sites in the tRNA structure react independently. The overall rates of modification of adenosine and cytidine residues in this tRNA were very similar (Figure 1). Reaction rates decreased at

[2] Previous chemical modification experiments on purified *E. coli* tRNA^{fMet} have shown that the same sites are modified at 25 and 37 °C; however, the reaction at 25 °C plateaus after modification of exposed bases, while that at 37 °C slowly continues, indicating some "breathing" of the structure.

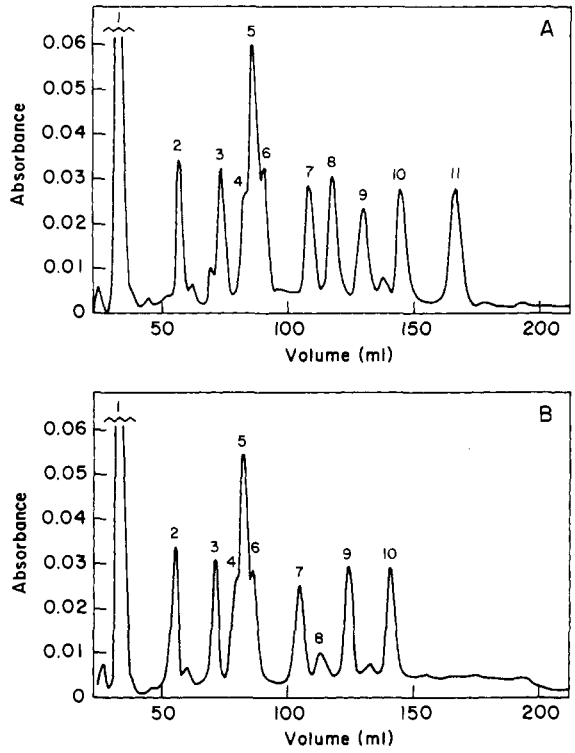


FIGURE 2: Fractionation of oligonucleotides obtained from RNase T_1 digestion of tRNA^{fMet} on RPC-5 at pH 7.4. (A) Unmodified tRNA^{fMet}; (B) tRNA^{fMet} treated with 1 M chloroacetaldehyde in 10 mM magnesium acetate, 20 mM potassium acetate, pH 6.0, at 25 °C for 8 h. Chromatography conditions are given under Materials and Methods. The two large oligonucleotides, T-Ψ-C-A-A-A-U-C-C-Gp and C^mU-C-A-U-A-A-C-C-C-Gp, did not completely separate from each other on this column and were isolated on DEAE-cellulose in 7 M urea as described in the text. The identities of the peaks are as follows: 1, Gp; 2, C-Gp; 3, A-Gp; 4, D-A-Gp; 5, U-C-Gp, m^7G-U-C-Gp; 6, C-A-Gp; 7, A-A-Gp; 8, C-C-U-Gp; 9, C-U-C-Gp; 10, C-C-C-C-C-Gp; 11, C-A-A-C-C-A.

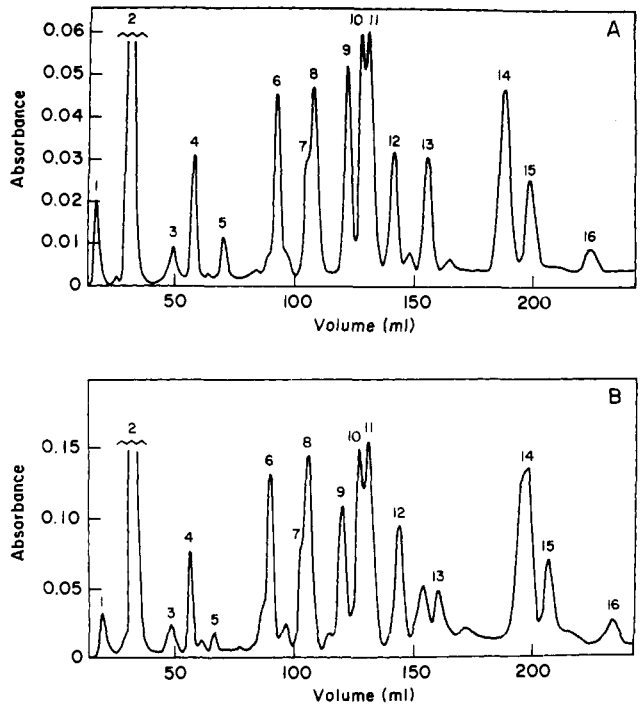


FIGURE 3: Fractionation of oligonucleotides obtained from pancreatic RNase digestion of tRNA^{fMet} on RPC-5 at pH 9.2. (A) Unmodified tRNA^{fMet}; (B) chloroacetaldehyde-modified tRNA^{fMet}. Reaction conditions were as described in Figure 2. Chromatography conditions are given under Materials and Methods. The identities of the peaks are as follows: 1, A; 2, Cp; 3, Ψp; 4, Up; 5, pCp; 6, G-Cp; 7, A-Up; 8, G-Up; 9, A-A-Cp; 10, A-G-Cp; 11, G-G-Cp, G-G-Dp; 12, G-G-Tp; 13, A-A-A-Up; 14, G-G-A-G-Cp, G-G-G-C^m-Up; 15, G-A-A-G-m^7G-Up; 16, G-A-A-G-A-Up.

longer reaction times; however, this appeared to be due to the instability of chloroacetaldehyde at the pH of the reaction, since addition of fresh reagent led to additional modification of the tRNA up to a total of 5 adenosine and 6 cytidine residues.

The adenosine derivative, εA, is a highly fluorescent compound (Barrio et al., 1972); thus, the reaction of adenosine residues in tRNA^{fMet} could also be followed by the linear increase in fluorescence emission at 410 nm on excitation at 305 nm. At neutral pH, the fluorescence emission spectrum of chloroacetaldehyde-modified tRNA^{fMet} closely corresponded to that of εA (Secrist et al., 1972), indicating that the fluorescence of εC makes little contribution to the spectrum under these conditions.

Location of Chloroacetaldehyde-Modified Residues in tRNA^{fMet}. The locations of εA and εC residues in chloroacetaldehyde-modified tRNA^{fMet} were determined following ribonuclease digestion and column chromatography. Figures 2 and 3 compare the oligonucleotide profiles obtained by chromatography on RPC-5 following digestion of modified and unmodified tRNA^{fMet} with RNase T_1 and pancreatic RNase. Loss of absorbance peaks clearly indicated extensive reaction of residues in certain oligonucleotides. These included C_{16}-C_{17}-U-Gp (peak 8, Figure 2), the 3′ terminal RNase T_1 fragment, C-A-A-C-C-A (peak 11, Figure 2), the 5′ terminal cytidine released by pancreatic RNase as pCp (peak 5, Figure 3), one or both of the A-A-Cp sequences in tRNA^{fMet} (peak 9, Figure 3), and the oligonucleotide A-A-A-Up (peak 13, Figure 3). Other modifications did not cause shifts in the oligonucleotide profiles and it was necessary to examine each fraction for the presence or absence of εA and εC. This analsis revealed that the pattern of cytidine modification by chloroacetaldehyde was the same as that previously observed in tRNA^{fMet} using several other chemical reagents (Goddard and Schulman, 1972; Chang, 1973; Schulman et al., 1974). The details of this analysis will therefore not be presented; however, it should be noted that repeated chromatography of εC-containing oligonucleotides resulted in progressive loss of the modified C derivative with formation of other unknown products. Thus, chloroacetaldehyde is less suitable for quantitative studies of exposed cytidine residues in tRNA than the cytidine-specific reagents which have previously been used.

Chloroacetaldehyde-modified adenosine residues in tRNA^{fMet} were found to be stable to the experimental manipulations required for structural analysis. The two large oligonucleotides, C^m-U-C-A-U-A-A-C-C-C-Gp and T-Ψ-C-A-A-A-U-C-C-Gp, obtained from RNase T_1 digestion of the modified tRNA were isolated by chromatography on DEAE-cellulose in 7 M urea as described previously (Schulman, 1970). Nucleoside analysis revealed that both oligonucleotides contained εA and that the anticodon fragment also contained εC (Table I). In order to determine the location of the modifications, C^m-U-C-A-U-A-A-C-C-C-Gp was digested with pancreatic RNase and the resulting oligonucleotides were isolated by chromatography on RPC-5. Table II summarizes the data obtained. In agreement with the previous report of Tolman et al. (1974), pancreatic RNase was found to cleave CεA but not εCεA sequences. All of the modifications were found in the anticodon nucleotides C_{35} and A_{36}. No modifi-

TABLE I: Nucleoside Analysis of RNase T_1 Oligonucleotides Isolated from Chloroacetaldehyde-Modified $tRNA^{fMet}$.

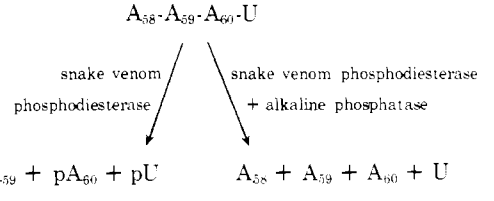
T-Ψ-C-A-A-A-U-C-C-Gp		C^m-U-C-A-U-A-A-C-C-C-Gp	
Nucleoside	mol/mol of Guanosine	Nucleoside	mol/mol of Guanosine
T	0.9		
Ψ	1.0	C^m	1.0
C	3.2	C	3.0
U	1.1	U	1.8
A	2.0	A	2.4
ϵA	0.6	ϵA	0.2
ϵC	0	ϵC	+

cation of adenosine residues 38 and 39 on the 3′ side of the anticodon loop could be detected.

The site of the modified adenosine residue in the TΨC loop of $tRNA^{fMet}$ was investigated by digestion of modified T-Ψ-C-A-A-A-U-C-C-Gp with pancreatic RNase and alkaline phosphatase and isolation of the resulting A-A-A-U by chromatography on RPC-5. The location of the modified adenosine residue was determined by nucleoside analysis following digestion of the oligonucleotide with purified snake venom phosphodiesterase in the presence and absence of alkaline phosphatase. In the absence of phosphatase, the only residue released as a nucleoside is A_{58} (see Scheme I). All of the ϵA present in the oligonucleotide was released by snake venom phosphodiesterase alone and was therefore present at position 58 (Table III).

The only other ϵA residues in chloroacetaldehyde-modified $tRNA^{fMet}$ were in the 3′ terminal RNase T_1 fragment, C-A-A-C-C-A. At the early stages of the chloroacetaldehyde reaction, the modified oligonucleotide appeared as a discrete peak eluting just after unmodified C-A-A-C-C-A. Continued reaction resulted in progressive broadening of this peak,

Scheme I



suggesting multiple modifications. After 8-h reaction, the oligonucleotide appeared to be completely lost from the RNase T_1 profile (peak 11, Figure 2); however, it was actually present n a very broad band eluting between 150 and 200 ml (Figure 2B). The modified oligonucleotide could be specifically identified as the 3′ terminal sequence, since this is the only RNase T_1 fragment which does not contain G. Complete enzymatic degradation of each fraction in this region of the chromatogram to nucleosides resulted in an approximately 12-fold increase in fluorescence intensity. This is to be compared with 1.4-fold increase on similar digestion of modified T-Ψ-C-A-A-A-U-C-C-Gp. These differences reflect both the extent of ϵA modification and the degree of fluorescence quenching due to intramolecular base–base interactions in different ϵA-containing oligonucleotides (Tolman et al., 1974).

The broad band of modified C-A-A-C-C-A was pooled and further digested with pancreatic RNase and alkaline phosphatase. The modified A_{73}-A_{74}-C isolated by this procedure was analyzed by nucleoside analysis following digestion of the oligonucleotide with purified snake venom phosphodiesterase

TABLE II: Pancreatic RNase Digestion Products of Modified C^m-U-C-A-U-A-A-C-C-C-Gp Isolated from Chloroacetaldehyde-Treated $tRNA^{fMet}$.

Product	mol/mol of Guanosine
ϵCp	*a*
Cp	*a*
C^m-Up	1.00
A-Up	0.83
ϵA-Up	0.12
ϵC-ϵA-Up	0.08
A-A-Cp	0.96

[a] The yields of Cp and ϵCp could not be quantitated due to the partial breakdown of ϵC during rechromatography to yield several unknown products, one of which cochromatographed with unmodified cytidine on nucleoside analysis. See Table I for the yield of cytidine in this oligonucleotide prior to rechromatography.

in the presence and absence of alkaline phosphatase as described before. The results given in Table III show that approximately half of the ϵA present in this sequence is at position 73 and half is at position 74. The amount of ϵA present at the 3′ terminus of $tRNA^{fMet}$ was taken as the difference between the total ϵA in C-A-A-C-C-A and the ϵA present in A_{73}-A_{74}-C.

The location and extent of chloroacetaldehyde modification of adenosine residues in $tRNA^{fMet}$ following treatment with 1 M chloroacetaldehyde at pH 6.0 for 8 h at 25 °C is summarized in Table IV.

Sites of Exposed Adenosine Residues in fMet-tRNA^{fMet}. Parallel modification experiments were carried out on $tRNA^{fMet}$ and fMet-$tRNA^{fMet}$ with 1 M chloroacetaldehyde, pH 5.5, at 25 °C in order to determine if any specific adenosine residues underwent a substantial change in reactivity following aminoacylation and formylation of the initiator tRNA. The locations of ϵA residues in oligonucleotides isolated from modified fMet-$tRNA^{fMet}$ were determined as described above. A_{36} was the only modified adenosine residue in the anticodon loop and A_{58} was the only modified adenosine residue in the TΨC loop in the formylated tRNA. Analysis of the 3′ terminal oligonucleotide showed that the total amount of ϵA, as well as the fraction at the 3′ terminus, was similar to that found in chloroacetaldehyde-modified $tRNA^{fMet}$ (Table V).

Bisulfite Modification of Uridine Residues in tRNA^{fMet}. Reaction of sodium bisulfite with tRNA at pH 7 provides a specific procedure for modification of uridine residues (Furuichi et al., 1970). No deamination of cytidine residues occurs under these conditions. The product of the reaction is a uridine-bisulfite adduct, which is reversible at pH 9 and stable at pH 6 (Shapiro et al., 1970; Hayatsu et al., 1970a,b).

Purified $tRNA^{fMet}_3$ was treated with 2 M sodium bisulfite, pH 7.0, in 10 mM $MgCl_2$ at 25 °C for 24 h. After removal of bisulfite, the modified tRNA was digested with RNase T_1 and the resulting oligonucleotides were chromatographed on RPC-5 at pH 6.0 (Figure 4). Loss of an oligonucleotide in the profile was found to result in the appearance of two new oligonucleotides eluting at higher salt concentration. The parent oligonucleotide could be obtained from either of the products by pH 9 treatment. The products therefore appear to represent oligonucleotides containing the two possible diastereomers of the uridine-bisulfite adduct. Further evidence for this was obtained by isolation of two isomeric products from a model compound, ApU (not shown).

TABLE III: Location of Adenosine Residues in Oligonucleotides Isolated from Chloroacetaldehyde-Modified tRNA^{fMet}.

Oligonucleotide		Treatment	Adenosine Residue Released as Nucleoside	nmol of Nucleoside	
Sequence	nmol			A	εA
A_{58}-A_{59}-A_{60}-U	1.7[a]	Snake venom phosphodiesterase	A_{58}	1.12	1.10
A_{58}-A_{59}-A_{60}-U	1.7[a]	Snake venom phosphodiesterase + phosphomonoesterase	A_{58} + A_{59} + A_{60}	3.69	1.03
A_{73}-A_{74}-C	2.8[b]	Snake venom phosphodiesterase	A_{73}	2.67	0.43
A_{73}-A_{74}-C	2.8[b]	Snake venom phosphodiesterase + phosphomonoesterase	A_{73} + A_{74}	4.68	0.84

[a] Based on total U. [b] Based on total (A + εA)/2.

TABLE IV: Summary of Chloroacetaldehyde Modification of Adenosine Residues in tRNA^{fMet}.

Residue	mol of εA/mol of tRNA
A_{36}	0.20
A_{58}	0.65
A_{73}	0.15
A_{74}	0.15
A_{77}	0.70

TABLE V: Comparison of εA Modifications in Chloroacetaldehyde-Treated tRNA^{fMet} and fMet-tRNA^{fMet}.

Oligonucleotide	Modified Residue	mol of εA/mol of tRNA	
		tRNA^{fMet}	fMet-tRNA^{fMet}
C^m-U-C-A-U-A-A-C-C-C-Gp	A_{36}	0.17	0.20
T-Ψ-C-A-A-A-U-C-C-Gp	A_{58}	0.65	0.60
3′ terminal A	A_{77}	0.25[a]	0.30[a]
C-A-A-C-C-A	A_{73} + A_{74} + A_{77}	0.38	0.43

[a] Data obtained by nucleoside analysis following pancreatic RNase digestion of the modified tRNA. The yield of εCεA$_{77}$ is not included.

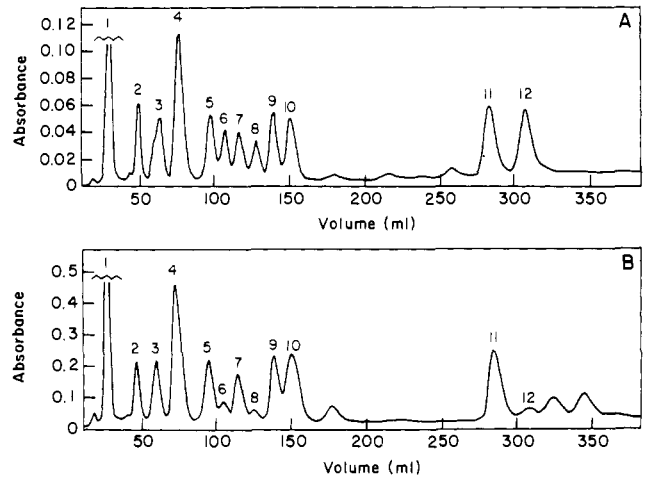


FIGURE 4: Fractionation of oligonucleotides obtained from RNase T_1 digestion of tRNA$^{fMet}_3$ on RPC-5 at pH 6.0. (A) Unmodified tRNA$^{fMet}_3$; (B) tRNA$^{fMet}_3$ treated with 2 M sodium bisulfite, pH 7.0, in 10 mM $MgCl_2$ at 25 °C for 24 h. Chromatography conditions are given under Materials and Methods. The identities of the peaks are as follows: 1, Gp; 2, C-Gp; 3, A-Gp; 4, D-A-Gp, U-C-Gp, C-A-Gp; 5, A-A-Gp, S-Gp; 6, C-C-U-Gp; 7, C-U-C-Gp; 8, A-U-C-Gp; 9, C-C-C-C-C-Gp; 10, C-A-A-C-C-A; 11, T-Ψ-C-A-A-A-U-C-C-Gp; 12, C^m-U-C-A-U-A-A-C-C-C-Gp.

Loss of peak 6, Figure 4, indicated extensive reaction of C-C-U_{18}-Gp in the dihydrouridine loop of tRNA^{fMet}. Modification of A-U_{48}-C-Gp (peak 8) indicated that the uridine residue in the small loop of tRNA$^{fMet}_3$ is exposed to attack by bisulfite. Similar experiments carried out on tRNA^{fMet} containing the sequence m^7G-U_{48}-C-Gp gave identical results (not shown). Modification of the anticodon loop was indicated by loss of peak 12 containing C^m-U-C-A-U-A-A-C-C-C-Gp. All three of the oligonucleotides which were altered following bisulfite treatment were fully modified in 24 h. The small amount of unmodified oligonucleotides appearing in the RNase T_1 profile is due to partial reversal of the uridine-bisulfite adducts during the ribonuclease digestion. In order to determine the relative rates of modification at each site, reactions were carried out for shorter times using [^{35}S]bisulfite. The results indicated that U_{37} and U_{48} were modified at similar rates and about three times faster than U_{18}. After 1.5-h reaction with 2 M bisulfite, pH 7 at 25 °C, the yields of modification (mol of ^{35}S/mol of tRNA) were 0.34 at U_{18}, 0.92 at U_{37}, and 0.96 at U_{48}. The extent of labeling at U_{34} vs. U_{37} was determined following pancreatic RNase digestion of modified C^m-U_{34}-C-A-U_{37}-A-A-C-C-C-Gp and chromatography on RPC-5. Pancreatic RNase readily cleaved after modified U_{37} giving rise to two well separated A-U_{37}-bisulfite adducts. Under conditions where U_{37} was almost fully modified, no loss of C^m-U_{34p} from the profile could be detected. After 48-h reaction with 2 M bisulfite at pH 7, it was found that a small amount of modification of U_{34} had occurred.

Discussion

E. coli tRNA^{fMet}, like yeast tRNA^{Phe}, is a class I tRNA (Levitt, 1969) containing four base pairs in the dihydrouridine stem and five bases in the variable loop. All class I tRNAs contain a number of invariant and correlated invariant residues in single-stranded regions of the clover leaf structure. Discussions of these features of tRNA structure can be found in the recent reviews by Sigler (1975) and Rich and Raj Bhandary (1976). Analysis of the crystal structure of yeast tRNA^{Phe} has shown that, except for the constant 3′ terminal CCA sequence, the invariant residues in this tRNA are involved in tertiary interactions which determine the three-dimensional shape of the molecule (Kim et al., 1974a; Robertus et al., 1974). It has been suggested that the stabilizing features provided by the constant bases are probably preserved in all class I tRNA structures (Kim et al., 1974b). Chemical modification experiments have shown that these bases are not accessible to attack in yeast tRNA^{Phe} (Robertus et al., 1974a).

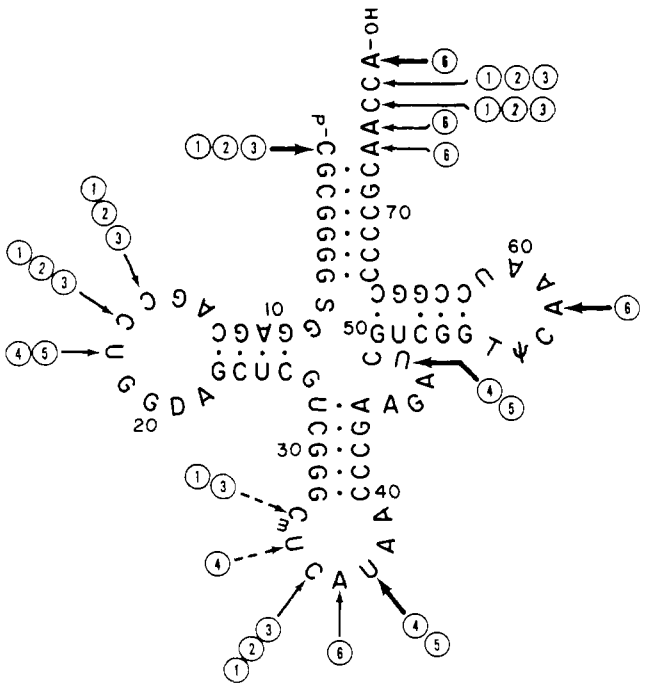


FIGURE 5: Summary of chemically reactive sites in *E. coli* $tRNA^{fMet}$. The primary structure shown here is $tRNA^{fMet}_3$. Heavy arrows indicate the residues most rapidly modified by a given reagent. Dashed arrows indicate sites weakly modified by one reagent and not significantly modified by another reagent (see text). (1) Bisulfite-catalyzed deamination, pH 6.0 (Goddard and Schulman, 1972); (2) methoxyamine, pH 5.5 (Chang, 1973); (3) bisulfite-catalyzed transamination, pH 7.0 (Schulman et al., 1974); (4) carbodiimide, pH 8.0 (Chang, 1973); (5) sodium bisulfite, pH 7.0 (this work); (6) chloroacetaldehyde, pH 6.0 (this work). Purified *E. coli* $tRNA^{fMet}_1$, having a m^7G residue at position 47 shows the same pattern of chemical modification as indicated above with reagents 1, 5, and 6.

In contrast, nucleotides in the anticodon loop, the two variable regions of the dihydrouridine loop, and the 3′ terminal C-C-A sequence are readily modified. In addition, the uridine residue in the variable loop is highly reactive (Robertus et al., 1974a).

The present work completes a similar study of the chemical reactivity of nucleotides in *E. coli* $tRNA^{fMet}$. The available data are summarized in Figure 5. The overall pattern of modification is very similar to that found in yeast $tRNA^{Phe}$, supporting the idea that many of the tertiary interactions in the two tRNAs are the same. Only the variable region in the dihydrouridine loop, containing C_{16}, C_{17}, and U_{18}, is chemically modified under nondenaturing reaction conditions. None of the guanosine residues in $tRNA^{fMet}$ are accessible to attack by carbodiimide, and only one nucleotide in the variable loop, U_{48}, can be chemically modified by any of the reagents tested. This uridine residue is also the only exposed nucleotide in the same region of yeast $tRNA^{Phe}$.

There are differences between the observed reactivity of nucleotides in the anticodon loops of the two tRNAs. In both cases, all of the anticodon bases are reactive; however, no modification of the adenosine residues on the 3′ side of the anticodon loop could be detected by chloroacetaldehyde-modification of *E. coli* $tRNA^{fMet}$, while these residues are modified by perphthalic acid in yeast $tRNA^{Phe}$ (Cramer et al., 1968). The reactivity of bases in the anticodon loop of tRNAs has been shown to be a function of the primary structure of the loop (Chang and Ish-Horowicz, 1974); however, it is also possible that differences may result from the particular reagent used.

The reactivity of uridine and cytidine residues in *E. coli* $tRNA^{fMet}$ has been investigated using several different reagents (see Figure 5). The data are in excellent agreement in all cases, except for the two residues on the 5′ side of the anticodon. No modification of $C_{33}{}^m$ was found following extensive treatment of $tRNA^{fMet}$ with methoxyamine at pH 5.5 (Chang, 1973), while a slow reaction was observed during sodium bisulfite catalyzed deamination at pH 6.0 (Goddard and Schulman, 1972) and transamination at pH 7.0 (Schulman et al., 1974). The adjacent uridine residue, U_{34}, reacted with carbodiimide at about one-fifth the rate of the anticodon base U_{37} (Chang, 1973). We have found the reactivity of these two residues with sodium bisulfite to differ by a factor of at least 50. No reaction of U_{33} could be detected under conditions leading to complete modification of U_{37} (2 h at 25 °C in 2 M HSO_3^-, pH 7) and partial reaction was observed only after several days reaction. Despite these differences, both sets of data indicate that the residues on the 5′ side of the anticodon of $tRNA^{fMet}$ have low reactivity to chemical reagents. It is not clear, however, whether this is an intrinsic property due to the environment of these bases or whether the more rapid reactions which occur in the anticodon sequence reduce the subsequent rate of modification of the other pyrimidines in the loop.

E. coli $tRNA^{fMet}$ has the unusual structural feature of an unpaired nucleotide at the 5′ terminus (Dube et al., 1968). The 5′ terminal cytidine is the most reactive cytidine in the molecule with all the reagents tested (see Figure 5) and the lack of a base pair in this position undoubtedly accounts for the reaction of the adjacent adenosine at position 73 with chloroacetaldehyde. This structural feature may also lead to a weaker base-stacking interaction of A_{74}, the fourth base from the 3′ terminus, which is modified by chloroacetaldehyde but unreactive with perphthalic acid in yeast $tRNA^{Phe}$ (Cramer et al., 1968).

The most interesting result to be obtained in the present work is the finding of a highly reactive adenosine residue in the middle of the seven-membered TΨC loop of *E. coli* $tRNA^{fMet}$. Yeast $tRNA^{Phe}$ contains a guanosine residue in this position, which is not chemically modified by reagents which attack two other guanosine residues in the dihydrouridine loop and the anticodon (Litt, 1971; Rhodes, 1975). The crystal structure of yeast $tRNA^{Phe}$ shows that the bases in the TΨC loop are extensively hydrogen bonded, interacting with each other and with residues in the dihydrouridine loop (Kim et al., 1974a; Ladner et al., 1975). The guanosine residue in the middle of the TΨC loop is intercalated between the base pairs G_{18}·Ψ_{55} and G_{19}·C_{56}. Recent high resolution data indicate that the amino N-2 of this guanosine residue is also hydrogen bonded to two ribose groups in the dihydrouridine loop (Ladner et al., 1975). This might account for its lack of modification by ketoxal, a reagent which requires both a free N-1 and amino N-2 for reaction. Lack of modification with carbodiimide, which attacks N-1, might be due to steric interference of the approach of this bulky reagent; however, Gamble and Schimmel (1974) have found that tritium exchange from ^3H_2O into the C-8 position of the guanosine residue in the TΨC loop is also much slower than the exchange rate observed for exposed purines in yeast $tRNA^{Phe}$. It therefore appears that there is a real difference in the chemical reactivity of the middle purine base of the TΨC loop in yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{fMet}$. Both N-1 and amino N-6 must be free for reaction of A_{58} in $tRNA^{fMet}$ with chloroacetaldehyde. The fact that this residue reacts at the same rate as the 3′ terminal adenosine residue and much faster than A_{36} in the anticodon suggests that A_{58} has only weak base stacking interactions, in contrast to the guanosine residue in the same position of yeast $tRNA^{Phe}$.

Since the TΨC loop has been implicated in ribosome binding of tRNAs, we investigated the possibility that this region of the initiator tRNA undergoes a conformational change which might be reflected in a difference in chemical reactivity following aminoacylation and formylation. No significant change was found in the location or extent of modification of any of the adenosine residues in fMet-tRNA^{fMet} compared to deacylated tRNA^{fMet}, however, indicating no major alteration in the environment of these residues following interaction of the tRNA with the aminoacyl synthetase and transformylase.

The question of whether the high reactivity of A_{58} in the TΨC loop of *E. coli* tRNA^{fMet} is a special property of the initiator tRNA remains open. Most class I tRNAs contain guanosine in the corresponding position and, like yeast tRNA^{Phe}, are almost completely resistant to chemical modification of the entire loop (Cramer, 1971). The only site which is clearly accessible is the N-1 position of the constant adenosine residue on the 3′ side of the loop. This site is methylated in vivo in many eukaryotic tRNAs and can be methylated in vitro in bacterial species, including *E. coli* tRNA^{fMet}, using heterologous methylating enzymes (Kuchino et al., 1971; Spremulli et al., 1974). Chemical methylation has also been found to occur at this site on treatment of yeast tRNA^{Ala} with dimethyl sulfate (Powers and Holley, 1972). Significantly, the adjacent guanosine residue in the middle of the TΨC loop was completely resistant to attack, even though the intrinsic reactivity of monomeric guanosine derivatives is greater than that of adenosine derivatives with this methylating reagent.

One other exception to the general resistance of bases in the TΨC loop of tRNAs to chemical modifications has recently been noted. Treatment of *E. coli* tRNA^{Ile} with an alkylating agent, chlorambucil, has been found to result in extensive modification of the TΨC loop in addition to the expected reaction in the anticodon and dihydrouridine loops (Wickstrom and Yarus, 1975). *E. coli* tRNA^{Ile}, like *E. coli* tRNA^{fMet}, is a class I tRNA containing an adenosine residue in the middle of the TΨC loop (Yarus and Barrell, 1971). Although the exact site of modification in the T-Ψ-C-A-A-Gp sequence of tRNA^{Ile} was not reported, the unusual results obtained with both *E. coli* tRNA^{fMet} and *E. coli* tRNA^{Ile} suggest the possibility that other class I tRNAs having an adenosine rather than a guanosine as the middle base of the TΨC loop may also show enhanced chemical reactivity of this region of the molecule.

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