Base substitutions in the wobble position of the anticodon inhibit aminoacylation of *E. coli* tRNA^{fMet} by *E. coli* Met-tRNA synthetase

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ABSTRACT.

Derivatives of *E. coli* tRNA^{fMet} containing single base substitutions at the wobble position of the anticodon have been enzymatically synthesized *in vitro*. The procedure involves excision of the normal anticodon, CAU, by limited digestion of intact tRNA^{fMet} with RNase A. RNA ligase is then used to join each of four trinucleotides, NAU, to the 5' half molecule and to subsequently link the 3' and modified 5' fragments to regenerate the anticodon loop. Synthesis of intact tRNA^{fMet} containing the anticodon CAU by this procedure yields a product which is indistinguishable from native tRNA^{fMet} with respect to its ability to be aminoacylated by *E. coli* methionyl-tRNA synthetase. Substitution of any other nucleotide at the wobble position of tRNA^{fMet} drastically impairs the ability of the synthetase to recognize the tRNA. Measurement of methionine acceptance in the presence of high concentrations of pure enzyme has established that the rate of aminoacylation of the AAU, GAU and UAU anticodon derivatives of tRNA^{fMet} is four to five orders of magnitude slower than that of the native or synthesized tRNA containing C as the wobble base. In addition, the inactive tRNA derivatives fail to inhibit aminoacylation of normal tRNA^{fMet}, indicating that they bind poorly to the enzyme. These results support a model involving direct interaction between Met-tRNA synthetase and the C in the wobble position during aminocylation of tRNA^{fMet}.

INTRODUCTION

Previous studies from this laboratory have used chemical modification methods to examine the structural requirements for recognition of *E. coli* tRNA^{fMet} by *E. coli* methionyl-tRNA synthetase (reviewed in ref. 1). These studies pointed to several specific regions of the tRNA as important for recognition by the enzyme. Particularly strong evidence indicated a role for the nucleotide base at the wobble position of the anticodon in the discrimination process.

T₄ RNA ligase has recently been used for the synthesis of normal and variant RNA sequences for a variety of structure-function studies (2-11). In this paper, we report the synthesis of tRNA^{fMet} molecules containing C,U,A and G in the wobble position of the anticodon using RNA ligase and examine the effect of base substitutions at this site on recognition of the tRNA by its cognate aminoacyl-tRNA synthetase.

EXPERIMENTAL PROCEDURES

Materials

Nucleoside 3' phosphates, nucleoside 5' diphosphates, nucleoside 3', 5'-bisphosphates, poly(A) and poly (A,U) were purchased from P.L. Biochemicals. $[5'\text{-}^{32}\text{P}]\text{rA}_{20}$ was prepared by the method of Silber *et al.* (12). Nucleoside 5' monophosphates and dinucleoside monophosphates were obtained from Sigma Chemical Company. $[\gamma\text{-}^{32}\text{P}]$ ATP, $[\alpha\text{-}^{32}\text{P}]$ ATP, and $[^{35}\text{S}]$ methionine were purchased from Amersham. *E. coli* tRNA^{fMet} having a specific activity of 1800 pmol per A₂₆₀ unit was obtained from Boehringer Mannheim. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* K₁₂ strain EM20031 as described before (13). T4 RNA ligase was purified from *E. coli* infected with T4 phage strain SP62, am N82 (14). The purified enzyme was free of detectable ribonuclease and had a specific activity of 1200 units/mg when assayed by the procedure of Moseman-McCoy *et al.* (15) using 10 μM $[5'\text{-}^{32}\text{P}]\text{rA}_{20}$ as substrate. T4 polynucleotide kinase (PNK) isolated from *E. coli* infected with T4 phage strain PseT 1 (16) was purchased from New England Nuclear. Primer dependent *M. luteus* polynucleotide phosphorylase, calf intestine alkaline phosphatase, and nuclease P1 were purchased from Boehringer Mannheim. Pancreatic RNase (RNase A) was obtained from Worthington and RNases T₁ and T₂ from Calbiochem. Purified rabbit liver tRNA nucleotidyltransferase was a gift from M. Deutscher.

Methods

Synthesis of oligonucleotides. The trinucleotides GpCpA and GpUpA were synthesized from the corresponding dinucleoside monophosphates and ADP using polynucleotide phosphorylase as described by Thach and Doty (17). GpApUp was synthesized by a similar reaction of GpA with UDP, but in the presence of 100 $\mu\text{g/ml}$ RNase A. The tetranucleotides GpCpApUp and GpUpApUp were synthesized by addition of pUp to the corresponding trinucleoside diphosphate using T4 RNA ligase (18). Treatment of the tetranucleotides with RNase T1 yielded the trinucleotides CAUp and UAUp. AAUp was isolated following digestion of poly(A,U) (1:1) with RNase A. All oligonucleotides were purified by chromatography on 0.5 x 100 cm columns of RPC-5 using a linear gradient of 0.075 - 1.6M ammonium acetate, pH 9.2 over 400 ml at a flow rate of 36 ml/hr. The samples were desalted by lyophilization. The base composition of each oligonucleotide was determined by total nucleoside analysis (19). Following removal of the 3' phosphate group with calf intestinal phosphatase, the 3' terminal nucleoside was identified by chromatography of a T2 RNase digest of the oligonucleotide on Aminex A-6 (20). The 5' terminal nucleotide was identified by labeling the oligonucleotide with $[\gamma\text{-}^{32}\text{P}]$ ATP and polynucleotide kinase, followed by digestion with nuclease P₁ and two-dimensional chromatography on cellulose thin-layer plates with unlabeled nucleoside 5' phosphate markers (21).

Preparation of tRNA^{fMet} half molecules. *E. coli* tRNA^{fMet} was dissolved in 0.1M Tris-HCl, pH 7.0, 5mM MgCl₂ at a concentration of 20 A₂₆₀/ml. The solution was incubated at 70° for 10 min., then at 37° for 30 min., and at 25° for 30 min. The solution was chilled and allowed to equilibrate at 4° for 1 hr. Pancreatic RNase (RNaseA) was added to a final concentration of 0.2 µg/ml and the solution incubated for 1 hr at 4°. The sample was quick-frozen in Dry Ice - acetone and lyophilized to absolute dryness. The residue was dissolved in 10M urea containing 0.2% xylene cyanole FF dye at a concentration of 250 A₂₆₀/ml. Samples were applied in 50 A₂₆₀ batches (200 µl) to 0.3 x 14 x 25 cm polyacrylamide slab gels containing 16% (w/v) acrylamide crosslinked with 1.3% (w/v) bisacrylamide in 0.1M Tris-borate buffer, pH8 and 8M urea. Electrophoresis was carried out at room temperature until the xylene cyanol dye had run to the bottom of the gel. The half molecule bands were detected by UV shadowing (22), excised with a sterile blade, and electrophoretically eluted from the gel slices (21). The samples were dialyzed vs 10mM Tris pH 7.5, 5mM MgCl₂, 50 mM NaCl, adjusted to a concentration of 20 A₂₆₀/ml and precipitated with 2 volumes of ethanol. The 5' half molecule fragment had a strong tendency to aggregate, especially in the absence of salt. The ethanol-precipitated fragment was therefore redissolved just before use in synthetic reactions.

Dephosphorylation of tRNA^{fMet} fragments was carried out in 0.1M Hepes, pH 8.3 at a fragment concentration of 20 A₂₆₀/ml by incubation with 3 units/ml calf intestinal phosphatase at 65° for 30 min. Solutions were adjusted to 8.3mM nitrilotriacetic acid (NTA), allowed to stand at room temperature for 20 min., and heated at 100° for 4 min. to inactivate the phosphatase (21). Samples were then adjusted to 25mM MgCl₂ and 125mM NaCl and precipitated by addition of 2 volumes of ethanol.

The fragments were identified by determination of the unique sequences which occur at the 3' and 5' ends of each half molecule. 5' Phosphorylation of each dephosphorylated fragment was carried out in 50 mM Hepes pH 8.3, 10 mM MgCl₂, 10 mM DTT, 0.1 mM [γ-³²P]ATP at a concentration of 1.7 A₂₆₀/ml by incubation with 800 units PNK/ml at 37° for 30 min. This 5' end labeling procedure gave a value of 3.5 nmoles fragment /A₂₆₀, which was subsequently used to calculate the concentration of unlabeled fragments. The 3' termini of each dephosphorylated fragment was labeled by addition of [5'-³²P] pCp to the 3' hydroxyl group using RNA ligase (23). Reaction mixtures contained 3.5 µM fragment, 10 µM [5'-³²P] pCp, 20 µM ATP and 100 µg/ml RNA ligase in 50mM Hepes, pH 7.5, 20 mM MgCl₂, 10 mM NaCl, 5 mM DTT and 10% DMSO. Incubations were for 16 hrs at 4°.

Nuclease digestions and chromatography of labeled fragments and synthetic tRNA products. Labeled tRNA fragments were isolated by polyacrylamide gel electrophoresis under denaturing conditions, autoradiography, and electrophoretic elution. For analysis of 5'-end labeled nucleotides, samples were taken up in 10 µl of

40mM ammonium formate buffer, pH 4.5 containing 1 unit of T2 RNase and incubated for 5 hrs at 37°. Samples were lyophilized, redissolved in 2 μ l of water containing 0.2A₂₆₀ of each nucleoside 3', 5'-bisphosphate and analyzed by two-dimensional chromatography on cellulose thin layer plates using isobutyric acid/0.5 N NH₄OH (5/3, v/v) as solvent for the first dimension and 0.1M sodium phosphate, pH 6.8/ammonium sulfate/isopropyl alcohol (100/60/2, v/w/v) as the second dimension (21). A similar procedure was used to analyze for the labeled 3'-terminal nucleotide of tRNA fragments, except that T2 RNase digested samples were chromatographed in the presence of unlabeled nucleoside 3' phosphate markers. Digests of 5' half molecule fragments also included unlabeled C^mpUp isolated by T2 RNase digestion of tRNA^{fMet}. Digestions of labeled tRNA fragments with nuclease P₁ were carried out in 10 μ l of 40 mM ammonium acetate, pH 6.0 containing 5 μ g of nuclease and incubated for 1-1/2 hrs at 50°. Samples were lyophilized and chromatographed in the presence of unlabeled nucleoside 5' phosphate markers as above.

Pancreatic RNase digestion of labeled fragments was carried out by incubation in 50 μ l of 0.1M Tris-HCl, pH 7.5 containing 2 A₂₆₀ of unlabeled poly (A,C) and 5 μ g RNase A for 18 hrs at 37°. Reaction mixtures were analyzed by chromatography on 0.5 x 100 cm columns of RPC-5 at a flow rate of 36 ml/hr using a linear gradient of 0.075 - 1.6M ammonium acetate, pH 9.2/400 ml. Absorbance patterns were recorded at 260 nm with a Gilford model 2400 absorbance recorder. Two ml fractions were collected and 1 ml aliquots counted in 15 ml of ACS II (Amersham). Labeled oligonucleotides were further identified by lyophilization and two dimensional thin layer chromatography with appropriate unlabeled markers, as discussed above. T₁ RNase digestion of labeled fragments was carried out by incubation in 150 μ l of 0.1 M Tris-HCl, pH 7.5 containing 2 A₂₆₀ of unlabeled tRNA^{fMet} and 35 units of T1 RNase for 2-1/2 hrs at 37°. In cases where calf intestinal phosphatase was included in the incubation, 0.3 units were added to the reaction mixture for the last hour. Digests were chromatographed on 0.5 x 100 cm columns of DEAE cellulose using a linear gradient of 0 - 0.45M NaCl/600 ml 0.02M Tris-HCl, pH 7.5, 7M urea at a flow rate of 15 ml/hr. Absorbance patterns were recorded, two ml fractions were collected, and radioactive peaks located as described above. Labeled oligonucleotides from 6 - 11 nucleotides long were desalted by dialysis in the presence of 2A₂₆₀ of carrier poly(A,C) or poly (A,U). Smaller oligonucleotides were desalted on 1 cm columns of DEAE cellulose equilibrated and washed with .02M ammonium bicarbonate, pH8. Oligonucleotides were eluted with 1M ammonium bicarbonate pH8 and further desalted by repeated lyophilization. Pancreatic RNase digestions of labeled T₁ RNase oligonucleotides were carried out as described above.

Synthesis of wobble base substituted tRNA^{fMet}. Equal amounts of the 3' and dephosphorylated 5' half molecules were mixed in 50 mM Hepes, pH 7.5, 20 mM MgCl₂

and 0.1 M NaCl to give a solution containing 175 nmoles/ml of each fragment. The half molecules were annealed by heating at 65° for 10 min and allowing the solution to slowly cool to room temperature over 1 hr. DTT was then added to a final concentration of 10 mM.

NpApUp triplets were phosphorylated at the 5' terminus in reaction mixtures which contained 1 mM oligonucleotide, 5 mM [γ -³²P] ATP, and 200 units/ml PseT 1 polynucleotide kinase in 50 mM Hepes, pH 7.5, 20 mM MgCl₂, and 10 mM DTT by incubation at 37° for 2 hrs. The kinase was inactivated by heating samples at 100° for 2 min.

Reannealed complex of 3' and 5' half molecules was added directly to the kinase reaction mixture for joining of the 5' phosphorylated triplet to the free 3' OH group of the 5' half molecules with RNA ligase. Reaction mixtures contained 70 μ M complex, 70 - 140 μ M oligonucleotide, 350 - 700 μ M ATP and 50 μ g/ml RNA ligase in 50 mM Hepes pH 7.5, 20 mM MgCl₂, 44 mM NaCl, and 10 mM DTT. Samples were incubated at 12° for 2 hrs, then heated at 65° for 5 min and electrophoresed on 0.3 x 14 x 25 cm polyacrylamide slab gels as described before. A₂₆₀ bands were detected by UV shadowing and the labeled product was located by autoradiography. The product (37 nucleotides) was well separated from the 5' fragment starting material (34 nucleotides), but ran at the leading edge of the 3' fragment (38 nucleotides) and was only partially resolved from it under our usual conditions of electrophoresis. The product and comigrating portion of the starting 3' fragment were excised and electrophoretically eluted together. The yield of elongated 5' fragment was calculated from the known specific activity of the ³²P label and the amount of unreacted 3' fragment was calculated from the total A₂₆₀ after subtracting the A₂₆₀ of the product. This procedure normally resulted in recovery of the elongated 5' fragment and unreacted 3' fragment in a ratio of 1:2.5 - 5. Eluted samples were dialyzed vs 10mM Tris, pH 7.5, 5 mM MgCl₂ and 50 mM NaCl, concentrated to 10-20 A₂₆₀/ml, and precipitated with 2 volumes of ethanol.

The mixture of fragments was dephosphorylated with calf intestinal phosphatase and reannealed at a concentration of 40 A₂₆₀/ml as described before. The fragments were phosphorylated at the 5' termini by incubation of 20 μ M reannealed complex (50 - 100 μ M 3' fragment) with 0.25 - 1mM [γ -³²P] ATP and 100 units/ml polynucleotide kinase in 50 mM Hepes, pH 7.5, 20 mM MgCl₂, 0.1 M NaCl, and 10 mM DTT at 37° for 1 hr. Joining of the 5' phosphorylated 3' fragment to the elongated 5' fragment was accomplished by addition of RNA ligase directly to the kinase reaction mixture to a final concentration of 9 μ g/ml and continued incubation at 37° for 30 min. The solution was heated at 65° for 5 min to inactivate the enzymes and the product precipitated by addition of 2 volumes of ethanol.

Enzymatic repair of the 3' terminal sequence of the joined fragments (5 - 10

μ M) was carried out in 50 mM glycine - NaOH, pH 9.2, 10 mM magnesium acetate, 1 mM DTT, 0.125 mM unlabeled CTP, and 0.5 mM [α - 32 P] ATP with 0.31 units/ml tRNA nucleotidyl transferase at 37° for 30 min. The final products were isolated by electrophoresis on denaturing polyacrylamide gels as described before, visualized by UV shadowing, excised, electrophoretically eluted, and dialyzed vs 10 mM Tris pH 7.5, 5 mM MgCl₂. The dialyzed samples were chromatographed on 1 cm columns of benzoylated DEAE cellulose in an attempt to remove non-nucleic acid A₂₆₀ which coeluted from the polyacrylamide gel, however, this procedure did not change the 32 P/A₂₆₀ of the samples. The synthesized products were stored at a concentration of 1500 nmoles/ml in 0.1 M Tris, pH 7.5, 10 mM MgCl₂.

RESULTS

Synthesis of tRNA^{fMet} containing base substitutions in the wobble position.

Limited digestion of tRNA^{fMet} with pancreatic RNase was used to generate half-molecule size fragments missing the anticodon nucleotides and the two terminal nucleotides of the CCA sequence. Considerable difficulty was encountered in consistently obtaining high yields of the desired fragments in early attempts, however the digestion and isolation procedures described under Methods and illustrated in Figure 1 reproducibly gave final recoveries of at least 50% of the starting tRNA as two unique half-molecule sized fragments (bands 2 and 3, Figure 1). Slightly better yields were obtained when the tRNA was heated and renatured immediately prior to digestion, and this procedure was adopted for routine preparation of the fragments. Analysis of the 3' and 5' termini of the fragments was carried out following dephosphorylation. The 5' terminus of each fragment was labeled with 32 P by incubation with polynucleotide kinase and [γ - 32 P] ATP. Digestion of kinased band 2, Figure 1 with T2 RNase yielded p*Ap. Digestion with pancreatic RNase yielded p*ApApCp. This sequence occurs only twice in tRNA^{fMet}, once at positions 37-39 and once at positions 72-74. Since it is found at the 5' end of a half molecule size fragment, it corresponds to positions 37-39 and indicates that the 3' half molecule is generated by a nucleolytic cleavage between U36 and A37. 5'- 32 P-Labeled band 3, Figure 1 yielded p*Cp on digestion with T2 RNase or RNase A and p*CpGp on digestion with T1 RNase. This sequence corresponds to the normal 5' terminus of intact tRNA^{fMet} and indicates that band 3 is the 5' half molecule.

The 3' termini of the two fragments were analyzed following addition of p*Cp to the 3'OH group with T4 RNA ligase. Digestion of the labeled 3' half molecule with pancreatic RNase yielded ApApCp*. This sequence corresponds to positions 72-74 in intact tRNA^{fMet} and indicates that two nucleotides have been removed from the 3' terminal CCA sequence. Digestion of the labeled 5' half molecule with T2 RNase yielded C^mpUp*. This sequence occurs only once in tRNA^{fMet}, at positions 32-33 in the

Figure 1. Limited digestion of tRNA^{fMet} with pancreatic RNase. Polyacrylamide gel electrophoresis of reaction mixtures containing 0.25 μ g/ml RNase A (lanes a,b,f and g) or 0.5 μ g/ml RNase A (lanes c,d,h, and i). Left (lanes a-d), preincubated tRNA; right (lanes f-i), no preincubation. Lanes e and j contain reaction mixtures incubated in the absence of RNase A.

anticodon loop. Thus the 5' half molecule is a 34-nucleotide fragment corresponding to residues 1-33 (using the numbering system recommended in ref. 24) and the 3' half molecule is a 37-nucleotide fragment corresponding to residues 37-74.

Derivatives of tRNA^{fMet} containing base substitutions in the wobble position of the anticodon were synthesized according to the procedure illustrated in Scheme 1. The dephosphorylated 5' half molecule was reannealed to the 3' half molecule containing a 3'P group. The trinucleotides p*NpApUp were joined to the 3'OH group of the 5' fragment using RNA ligase. Yields in this step were 15-25% depending on the oligonucleotide sequence. Significantly lower yields were obtained when joining reactions were carried out in the absence of the reannealed 3' fragment. Longer incubations or higher concentrations of RNA ligase led to formation of more of the desired products, however other side reactions became significant and difficulties were encountered in purification from derivatives of similar size. The conditions described under Methods were adopted in order to maximize the purity of the desired product. We have not investigated the side reactions in detail, but believe that they correspond to reverse reactions of RNA ligase similar to those recently described by Krug and Uhlenbeck (25). Under the

Scheme I. Steps in the synthesis of tRNA^{fMet} molecules containing base substitutions in the wobble position of the anticodon

conditions described in Methods, the isolated products HO-C₁—C^mUp*N₃4AUp were at least 90% pure. Figure 2 shows nearest neighbor analysis of the addition products containing A, C, G and U at position 34.

The extended 5' fragment and the 3' fragment were dephosphorylated at the 3' termini and phosphorylated at the 5' termini with polynucleotide kinase. The anticodon loop was joined by incubation with low concentrations of RNA ligase. Under these conditions, there were no detectable covalent reactions at the 3'OH of the 3' fragment or the 5'P of the 5' fragment whereas there was a rapid and nearly quantitative closure of

Figure 2. Nearest neighbor analysis of products obtained following ligation of ^{32}P -labeled triplets to the 5' half molecule. Two dimensional thin layer chromatography of nuclease P₁ digests (top) and T₂ RNase digests (bottom) of products obtained by addition of p*AAUp (far left), p*CAUp (second from left), p GAUp (second from right), and p UAUp (far right) to the fragment HO-C₁I—C^mU₃₃-OH.

Figure 3. Joining of the 5' fragment $pC_1-C^{m}Up*UAU-OH$ to the unlabeled 3' fragment $pA_{37}-AC_{74}-OH$ in the anticodon loop with RNA ligase to give intact tRNA size product. Lane a, no ligase. Lanes b,c, and d, incubated for 1 min, 5 min, and 10 min at 37° with 9 $\mu g/ml$ RNA ligase. The arrows indicate the positions of intact $tRNA^{fMet}$ (1), 37-nucleotide 3' fragment (2) and 34-nucleotide 5' fragment (3).

the anticodon loop. Figure 3 illustrates the joining of $pC_1-C^{m}Up*UAU_{36}-OH$ to unlabeled $pA_{37}-AC_{74}-OH$. The labeled 5' half molecule is converted to a tRNA - size product within 5 min at 37° .

Joining reactions were normally carried out using 5' and 3' half molecules labeled with ^{32}P at the 5' terminus in order to more readily analyze the purity of the product (see below). The final step in the synthesis was the enzymatic repair of the 3' terminal CCA sequence using tRNA nucleotidyl transferase. This reaction was carried out with unlabeled CTP and $[\alpha-^{32}P]ATP$ in order to more readily analyze the efficiency of the repair process. In all cases, the reaction was quantitative. The final products ($0.1 - 0.4 A_{260}$) were isolated from polyacrylamide gels. All of the products migrated in the same position as intact $tRNA^{fMet}$.

Structural analysis and purity of $tRNA^{fMet}$ derivatives.

The 3' and 5' termini and internal ligation sites were analyzed using the ^{32}P labels incorporated into the structure during the synthesis. All samples yielded

radioactive products which comigrated with the normal 5' and 3' terminal oligonucleotides pCGp and CAACCPA-OH, on digestion with T1 RNase. The ^{32}P label at the 5' terminus was the only one which was sensitive to phosphomonoesterase, being converted to inorganic phosphate. The ^{32}P label in the 3' terminal sequence was the only one which did not shift its elution position when phosphomonoesterase was added to T1 RNase digestions prior to chromatography on DEAE cellulose. In addition, this radioactive peak was missing from the profile of derivatives which had not been treated with tRNA nucleotidyl transferase, CTP and $[\alpha\text{-}^{32}\text{P}]$ ATP. The internal sites were analyzed by isolation of the T1 RNase oligonucleotides containing the anticodon sequence. Intact tRNA^{fMet} yields only two large oligonucleotides on digestion with T1 RNase: a 10 mer derived from the T Ψ C loop and an 11 mer derived from residues 32-42 containing the anticodon loop and four adjoining nucleotides of the anticodon stem. Synthetic tRNA^{fMet} derivatives containing A, C, and U in the wobble position yielded 3 labeled peaks when digested with T1 RNase plus phosphomonoesterase. Figure 4A shows the profile obtained from the derivative containing A₃₄. Peaks 1 and 2 correspond to $^{32}\text{P}_i$ derived from the 5' terminus of the molecule and the normal 3' terminal oligonucleotide CAACCPA-OH. Peak 3 comigrates with the normal 11-residue long oligonucleotide containing the anticodon sequence. Further digestion of this peak with pancreatic RNase yields two labeled oligonucleotides, C^mUp* and AAUp*, in a 1:1 ratio (Figure 4B). The ratio of the ^{32}P label in T1 RNase peaks 1, 2 and 3 is 0.7:1.0:2.0. This indicates that only 70% of the 5' terminal phosphate group of the intact tRNA has been replaced by polynucleotide kinase. Similar results were obtained with the other derivatives. Structural analysis of these derivatives is summarized below:

The tRNA^{fMet} derivative containing G as the wobble base gave a different profile of ^{32}P -labeled oligonucleotides following digestion with T1 RNase (Figure 5A). Peaks 2 and 3 comigrated with the normal 3' and 5' terminal oligonucleotides CAACCA-OH and pCGp and gave the expected labeled nucleotides when analyzed as described in Methods. Peak 1 migrated in the position of a trinucleotide and Peak 4 in the position of an octanucleotide. Further digestion of Peak 1 with pancreatic RNase yielded C^mUp* as the only labeled product (Figure 5B). Since the original oligonucleotide was derived from a T1 RNase digest, it must have had a G residue at the 3' end and the sequence of Peak 1

Figure 4. Structural analysis of $\text{tRNA}^{\text{fMet}}$ derivative containing A in the wobble position of the anticodon. A: T_1 RNase plus phosphomonoesterase digest of the product $\text{p}^*\text{C}_1\text{---C}^{\text{m}}\text{U}_{33}\text{p}^*\text{AAUp}^*\text{A}_{37}\text{---CCp}^*\text{A}_{76}\text{---OH}$ chromatographed on DEAE cellulose in the presence of unlabeled $\text{tRNA}^{\text{fMet}}$ oligonucleotides. Numbers above the arrows indicate the size of some marker oligonucleotides. No further radioactivity was eluted with a 0.45 M salt wash. B: Pancreatic RNase digestion of ^{32}P -labeled peak 3 from Fig. 4A chromatographed on RPC-5 in the presence of A_{260} marker oligonucleotides. High salt (1.6 M) wash was started at fraction 79.

must be $\text{C}^{\text{m}}\text{Up}^*\text{Gp}$. Peak 4 Figure 5A gave AUp^* as the only labeled product on further digestion with pancreatic RNase. The only labeled phosphate unaccounted for is the one derived from $\text{p}^*\text{A}_{37}\text{---AC}_{74}\text{---OH}$, which gave Up^* as its nearest neighbor following anticodon loop closure (not shown). Peak 4, Figure 5A is therefore assumed to be the expected octanucleotide $\text{AUp}^*\text{AACCCGp}$. A summary of the labeled oligonucleotides

Figure 5. Structural analysis of tRNA^{fMet} derivative containing G in the wobble position of the anticodon. A: T₁ RNase digest of the product p*C₁—C^mU₃₃p*GAUp*A₃₇—CCp*A₇₆-OH chromatographed on DEAE cellulose. B: Pancreatic RNase digest of ³²P-labeled peak 1 from Fig. 5A chromatographed on RPC-5. C: Pancreatic RNase digest of ³²P-labeled peak 4 from Fig. 5A chromatographed on RPC-5. No further radioactivity was eluted with a 0.45 M (A) or 1.6 M (B and C) salt wash.

obtained from this tRNA^{fMet} derivative is given below:

Figure 6. Aminoacylation of wobble base substituted $\text{tRNA}^{\text{fMet}}$ derivatives (40 nM) with purified *E. coli* methionyl-tRNA synthetase (0.4 nM). Control $\text{tRNA}^{\text{fMet}}$, $\text{O}—\text{O}$; CAU derivative, $\text{X}—\text{X}$; AAU (or GAU or UAU) derivative, $\bullet—\bullet$. Incubations were at 26° under conditions described in ref. 26.

The ratio of peaks 1,2,3, and 4 in Figure 5 is 1.0:1.0:0.7:1.0. This indicates that, as with the other $\text{tRNA}^{\text{fMet}}$ derivatives, only 70% of the 5' terminal phosphate group has been replaced.

Based on the ^{32}P labels, all of the products are approximately 90% pure. When the amount of product calculated from the ^{32}P label is compared with the UV absorbance, specific activities of 800-1000 pmoles/ A_{260} are obtained. Similar specific activities are obtained for equivalent amounts of intact $\text{tRNA}^{\text{fMet}}$ following electrophoresis and isolation from polyacrylamide gels due to contamination of small samples with non-dialyzable UV absorbing material from the gels. The ^{32}P -label has therefore been used to calculate the actual concentration of each product for aminoacylation studies.

Aminoacylation of $\text{tRNA}^{\text{fMet}}$ derivatives containing wobble base substitutions.

Aminoacylation of the synthesized $\text{tRNA}^{\text{fMet}}$ derivative containing C as the wobble base (normal anticodon) was indistinguishable from that of control $\text{tRNA}^{\text{fMet}}$ under our usual assay conditions (26) using purified methionyl-tRNA synthetase. One mole of methionine was accepted per mole of synthetic product at a rate comparable to that observed with native $\text{tRNA}^{\text{fMet}}$ (Figure 6). The synthetic derivative differs from the control tRNA only in that 30% of the molecules are missing the 5' terminal phosphate group. Previous studies have shown that the dephosphorylated tRNA is aminoacylated to the same extent as normal $\text{tRNA}^{\text{fMet}}$ (27) and no differences have been observed in the rate of aminoacylation of synthesized tRNA derivatives depending on the extent of phosphorylation of the 5' terminus in the present studies.

TABLE I
Aminoacylation of Synthesized tRNA^{fMet} Derivatives
Containing Wobble Base Substitutions

Anticodon Sequence	tRNA (nM)	MetRS (nM)	Moles of methionine accepted per mole of tRNA	Incubation Time (min)	Relative Rate of Aminoacylation
CAU	40	0.4	1.0	5	1.0
	40	4.	1.0	1	
	820	148.	1.0	1	
AAU	40	4.	< 0.01	30	< 10 ⁻⁵
	40	40.	< 0.01	30	
	40	400.	< 0.01	30	
	405	62.	< 0.01	30	
	950	148.	< 0.01	30	
GAU	40	4.	< 0.01	30	< 10 ⁻⁵
	40	40.	< 0.01	30	
	40	400.	< 0.01	30	
UAU	40	4.	< 0.01	30	< 10 ⁻⁴
	40	40.	< 0.01	30	
	40	400.	0.01	30	

There was no detectable methionine acceptance by the synthesized tRNA^{fMet} derivatives containing A,G, or U in the wobble position under conditions leading to complete aminoacylation of the C derivative within 5 min. Further investigation of the aminoacylation of the inactive tRNAs using stoichiometric amounts of purified synthetase and 30 min incubation times also failed to yield detectable methionine incorporation (Table I). In the presence of a 10-fold excess of pure enzyme, the tRNA^{fMet} derivative containing U₃₄ showed a low level of methionine acceptance. These data indicate that tRNA^{fMet} containing any nucleotide base other than C in the wobble position is aminoacylated at a rate which is at least 4 to 5 orders of magnitude slower than the rate observed with the synthesized derivative having a normal anticodon sequence.

The ability of the defective tRNA derivatives to compete with normal tRNA^{fMet} during aminoacylation was also investigated. Under conditions of limiting enzyme concentration (13), the rate of aminoacylation of control tRNA samples was not inhibited by the presence of a 10-fold excess of the inactive tRNAs. Due to a shortage of the synthesized samples, higher concentrations could not be tested, however, the results indicate that the defective tRNAs are significantly impaired in their ability to be recognized by the synthetase, as well as in their ability to be aminoacylated.

DISCUSSION

The data presented in this paper confirm and extend our previous observations on the effect of alterations in the wobble position on aminoacylation of tRNA^{fMet} (1, 28, 29). The cytidine residue of the anticodon has been shown to be an essential base for recognition of methionine tRNAs by *E. coli* Met-tRNA synthetase. Anticodon base substitutions have also been found to cause dramatic decreases in the rates of aminoacylation of *E. coli* tRNA^{Gly} (30) and yeast tRNA^{Val} (31), and a dramatic enhancement in the rate of misacylation of Su⁺7 tRNA^{Trp} with glutamine (32). More modest effects of base substitutions on recognition of yeast tRNA^{Phe} (3) and *E. coli* tRNA^{Trp} (32) by cognate enzymes have also been noted.

The available evidence supports a model in which there is direct interaction between *E. coli* MetRS and the wobble base of substrate methionine tRNAs. The role of other anticodon bases in recognition by the enzyme remains unclear. Synthesis of tRNAs containing altered anticodon structures with T4 RNA ligase is an attractive means of investigating the nucleotide sequence requirements, and possibly even the functional group requirements, in this region for aminoacylation by both cognate and noncognate synthetases. Results of such studies on the effects of alterations at other sites in the anticodon of tRNA^{fMet} will be reported elsewhere.

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