Escherichia coli Dimethylallyl Diphosphate:tRNA Dimethylallyltransferase: Essential Elements for Recognition of tRNA Substrates Within the Anticodon Stem−Loop[†]

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ABSTRACT: *Escherichia coli* dimethylallyl diphosphate:tRNA dimethylallyltransferase (DMAPP−tRNA transferase) catalyzes the alkylation of the exocyclic amine of A37 by a dimethylallyl unit in tRNAs with an adenosine in the third anticodon position (position 36). By use of purified recombinant enzyme, steady-state kinetic studies were conducted with chemically synthesized RNA oligoribonucleotides to determine the essential elements within the tRNA anticodon stem−loop structure required for recognition by the enzyme. A 17-base oligoribonucleotide corresponding to the anticodon stem−loop of *E. coli* $tRNA^{Phe}$ formed a stem−loop minihelix ($minihelix^{Phe}$) when annealed rapidly on ice, while the same molecule formed a duplex structure with a central loop when annealed slowly at higher concentrations. Both the minihelix and duplex structures gave k_{cat}s similar to that for the normal substrate (full-length $tRNA^{Phe}$ unmodified at A37), although the K_m for $minihelix^{Phe}$ was approximately 180-fold higher than full-length tRNA. The A36-A37-A38 motif, which is completely conserved in tRNAs modified by the enzyme, was found to be important for modification. Changing A36 to G in the minihelix resulted in a 260-fold reduction in k_{cat} compared to $minihelix^{Phe}$ and a 13-fold increase in K_m. An A38G variant was modified with a 9-fold reduction in k_{cat} and a 5-fold increase in K_m. A random coil 17-base oligoribonucleotide in which the loop sequence of *E. coli* $tRNA^{Phe}$ was preserved, but the 5 base pair helix stem was completely disrupted and showed no measurable activity, indicating that a helix−loop structure is essential for recognition. Finally, altering the identity of several base pairs in the helical stem did not have a major effect on catalytic efficiency, suggesting that the enzyme does not make base-specific contacts important for binding or catalysis in this region.

Transfer RNA (tRNA) is the substrate for a number of enzymes catalyzing reactions that modify the structure of the four standard RNA nucleosides (reviewed in refs *1* and *2*). The modifications occur at various stages of tRNA maturation after synthesis of the tRNA transcript. Modified bases are located throughout the tRNA molecule, but the greatest variety is found in the anticodon loop. It has been estimated that as much as 1% of the bacterial genome is devoted to tRNA modification (*3*).

A modification found in tRNA from virtually all species of bacteria and eukarya is (dimethylallyl)adenosine 37 (i^6A)[1,2] and its thiomethylated (ms^2i^6A) and hydroxylated (*cis*-ms^2io^6-A) derivatives (Scheme 1). During formation of i^6A, the 5-carbon dimethylallyl moiety from dimethylallyl diphosphate (DMAPP) is transferred to the free amine of adenosine at position 37, immediately 3′ to the anticodon in tRNAs. This modification occurs in tRNAs with an adenosine at position 36, the third anticodon base, which read codons beginning with U. The prenyl transfer reaction is the first in a series of modifications to A37 that are proposed to proceed in the order A37 → i^6A37 → ms^2i^6A37 → *cis*-ms^2io^6A37 (3) (Scheme 1). The extent of modification at A37 depends on the organism. In *Escherichia coli*, fully modified tRNA contains ms^2i^6A (*4*), while in many other bacteria the modified nucleotide is *cis*-ms^2io^6A (*5*−*8*). Almost all eukaryotes have i^6A37 in mature tRNA, except for $tRNA^{Phe}$, in which a G at position 37 is modified to wybusine. Bacterial tRNAs from *Mycoplasma* and tRNAs from organisms in the domain Archaea are completely lacking in i^6A and its derivatives (*1*).

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[1] Abbreviations: BME, 2-mercaptoethanol; BSA, bovine serum albumin; *cis*-ms^2io^6A, *cis*- 2-(methylthio)-N^6-(4-hydroxydimethylallyl)-adenosine; DMAPP, dimethylallyl diphosphate; DMAPP−tRNA transferase, dimethylallyl diphosphate:tRNA dimethylallyltransferase; EDTA, ethylenediaminetetraacetic acid; i^6A, N^6-(dimethylallyl)adenosine; L, lysidine; m^2G, 2-methylguanosine; ms^2i^6A, 2-(methylthio)-N^6- (dimethylallyl)adenosine; PAGE, polyacrylamide gel electrophoresis; t^6A, N^6-(threoninylcarbonyl)adenosine; TCA, trichloroacetic acid; TE, Tris/EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0); Tris, Tris-(hydroxymethyl)aminomethane hydrochloride.

[2] The commonly used name N^6- (Δ^2-isopentenyl)adenosine, or i^6A, is incorrect for this modified nucleoside. In modern organic nomenclature the term isopentenyl refers to a 3-methyl-3-butenyl unit, while the moiety attached to the adenosine is a 3-methyl-2-butenyl, or dimethylallyl, unit. Therefore, the modified base is referred to herein as (dimethylallyl)adenosine. Since the dimethylallyl unit is a simple five-carbon isoprenoid moiety, we will continue to use the abbreviation i^6A to avoid confusion with previous literature in this area.

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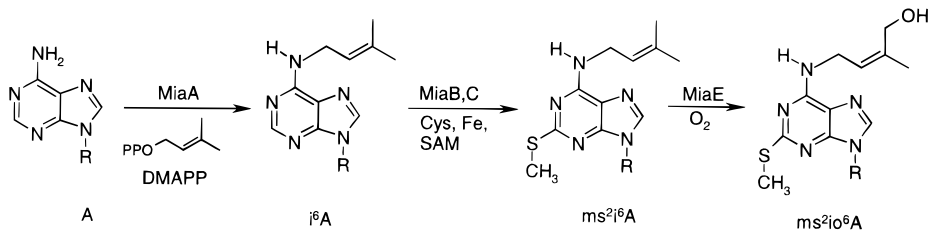
Scheme 1: Biosynthesis of i^6A and Its Derivatives[a]



[a] MiaA, MiaB, MiaC, and MiaE are the gene products responsible for catalysis of the indicated reactions.

The functions of the i^6A-based modifications have been extensively studied. The absence of i^6A modifications appears to have no direct kinetic effect upon aminoacylation (*9*, *10*). However, i^6A promotes binding of tRNA to the ribosome (*10*) and enhances weak A−U base pairing at the critical first base of the codon by stabilizing interstrand and intrastrand base stacking (*11*, *12*). In some cases the latter effect influences the expression of operons involved in amino acid biosynthesis (*13*). Recently, increasing evidence has pointed to the role played by i^6A and other tRNA modifications in cellular responses to environmental stress (*14*).

Transfer of the dimethylallyl unit to A37 is catalyzed by DMAPP-tRNA transferase, encoded by the *miaA* gene in bacteria and *MOD5* in *Saccharomyces cerevisiae*. Previously, DMAPP−tRNA transferase had been obtained from a number of sources (*15*−*21*). Rosenbaum and Gefter (*21*) purified the *E. coli* enzyme 550-fold and established that the enzyme required a divalent cation and β-mercaptoethanol (BME) for maximum activity and had a pH optimum of 7.5. Kline et al. (*20*) purified yeast DMAPP−tRNA transferase 50−100-fold and found similar behavior. Recently, two groups have described kinetic studies of purified recombinant DMAPP−tRNA transferase (*22*, *23*). Moore and Poulter (*22*) reported that the enzyme is a monomer of 34.7 kDa and that the kinetic mechanism is ordered sequential with tRNA binding before DMAPP; $k_{cat} = 0.48\ s^{-1}$, $K_m^{DMAPP} = 3.2\ \mu M$, and $K_m^{tRNA} = 96$ nM for $tRNA^{Phe}$ lacking the i^6A modification. Later, pre-steady-state experiments gave a lower value of $K_m^{tRNA} = 26.7$ nM (*24*). Leung et al. (*23*) reported similar kinetic parameters, except for $K_m^{tRNA} = 2$ nM. They also proposed that the enzyme was a monomer in solution but bound its tRNA substrate as a multimer.

Comparisons of tRNA sequences that are modified by DMAPP−tRNA transferase show several highly conserved features within the anticodon stem−loop, including an A36-A37-A38 motif, G−C base pairs at positions 29−41 and 30−40, and lack of G−C or C−G at position 31−39 (Figure 1). Motorin et al. (*25*) examined 17 variants of *E. coli* $tRNA^{Ser}$ (GGA) altered primarily in the anticodon stem−loop of the tRNA molecule for DMAPP−tRNA transferase activity using a crude cell extract from *E. coli*. Substrate tRNAs were generated by runoff transcription with [α-^{32}P]ATP, and modified AMP was quantified after resolution by cellulose thin-layer chromatography. The authors concluded that the enzyme requires an A36-A37-A38 motif for activity. In addition, they reported a strong preference for G−C base pairs at positions 29−41 and 30−40 in the anticodon stem. Reversing the bases to a C−G pair at either of those positions resulted in severely inhibited activity. However, the use of crude cellular extracts to assess activity limited the authors to a qualitative assessment of the tRNAs as active, inactive, or marginally active substrates for modification. Another limitation of this study is the fact that the modified base in *E. coli* is actually ms^2i^6A (*5*), with the thiomethylation step occurring after formation of i^6A and putatively requiring two additional enzymes, neither of which has yet been characterized (*1*, *26*). Variations in the sequence of the tRNA substrate would presumably affect the activity of these enzymes as well as DMAPP−tRNA transferase, thus complicating interpretation of the results.

The availability of pure recombinant DMAPP−tRNA transferase has permitted us to gain a quantitative understanding of the effects of changing putative recognition elements in the tRNA substrate, by directly determining the steady-state kinetic parameters for the reaction with altered substrates. We have used as substrate analogues several 17-base oligoribonucleotides corresponding to the stem-loop structure of $tRNA^{Phe}$. The specific bases studied were A36, A38, the G−C base pairs at positions 29−41 and 30−40, and the A−U or U/Ψ−A pair at position 31−39. We also examined a 17-mer where the stem was completely disrupted in order to determine if the A36-A37-A38 motif alone was sufficient for recognition.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. Oligoribonucleotides were synthesized in the Utah Regional Cancer Center Protein/DNA Core Facility. The solid samples were dissolved in 10 mM Tris/1 mM EDTA, pH 8.0 (TE), dialyzed against 1 L of TE for 6 h (molecular weight cutoff = 3500), and precipitated overnight at −78 °C following addition of 1.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2. The precipitate was pelleted by centrifugation, the supernatant was removed, and the RNA pellet was allowed to air-dry for 5 min. Samples were then redissolved in TE buffer, and concentrations were measured by absorbance at 260 nm using the sum of the extinction coefficients of the constituent bases. The oligoribonucleotides were pure after this treatment as judged by electrophoresis on a 20% polyacrylamide gel. [1-^{3}H]DMAPP was purchased from Dupont−NEN. Cold DMAPP was synthesized by the method of Davisson et al. (*27*). Stock solutions of DMAPP were prepared in 25 mM NH_4HCO_3, and the concentration of DMAPP was determined by phosphate analysis (*28*). $tRNA^{Phe}$ unmodified at A37 and *E. coli* DMAPP−tRNA dimethylallyltransferase were obtained as described by Moore and Poulter (*22*).

Steady-State Kinetic Measurements. RNA was dissolved in TE buffer containing 7 mM $MgCl_2$. The solutions were placed in boiling water for 5 min and then cooled rapidly on ice. The concentration of RNA used in the annealing

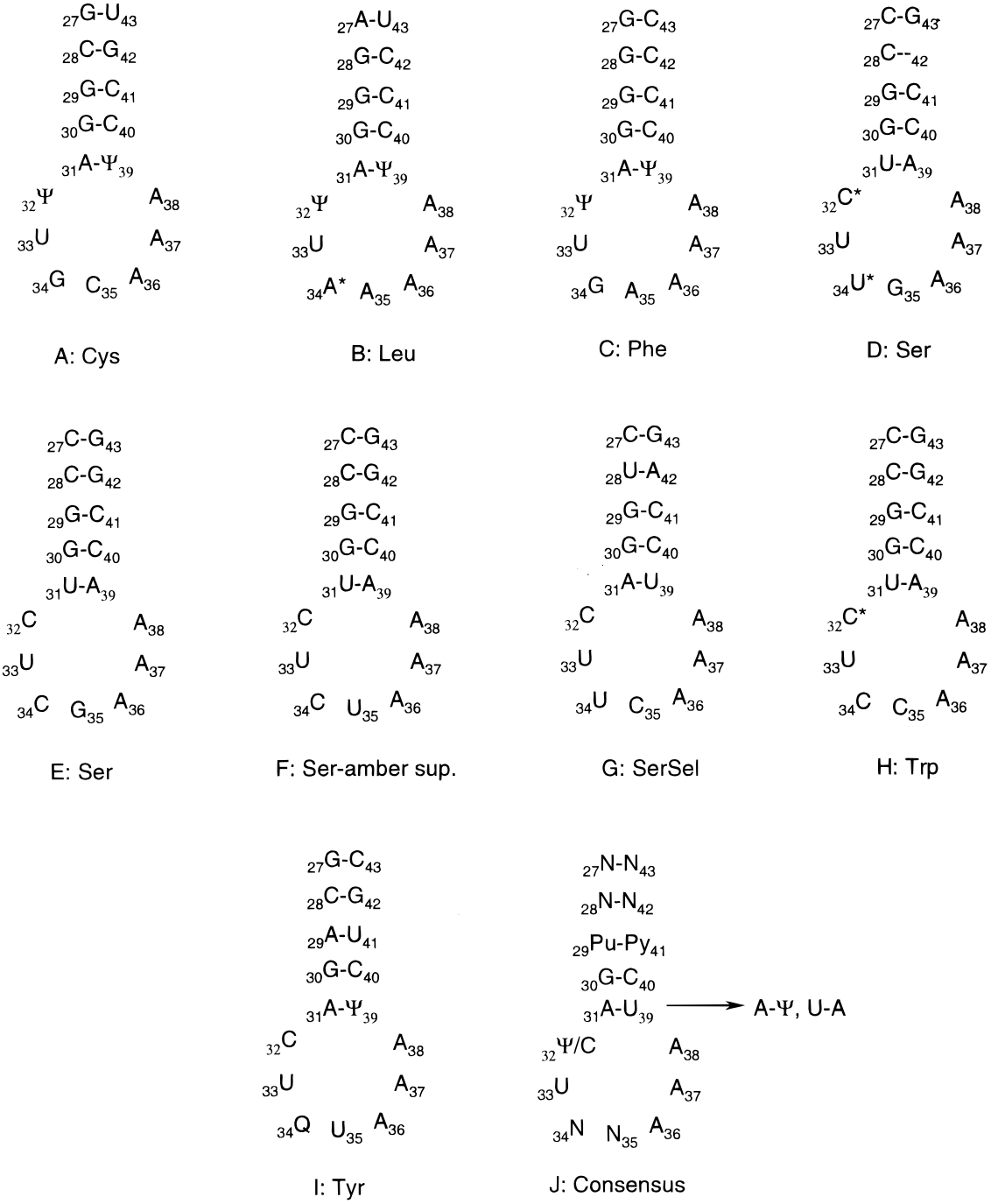


FIGURE 1: Sequences of the anticodon stem−loops of *E. coli* tRNAs that are modified by DMAPP−tRNA transferase. Ψ is pseudouridine, Q is queuine, and N is any nucleotide. (A) $tRNA^{Cys}$ (*35*). (B) $tRNA^{Leu}$ (*36*). (C) $tRNA^{Phe}$ (*37*). (D) $tRNA^{Ser}$ (UGA), position 32 is 2′-*O*-methyl C, position 34 is 5-hydroxy-U (*38*). (E) $tRNA^{Ser}$ (CGA) (*39*). (F) Amber suppressor $tRNA^{Ser}$ (*40*). (G) Selenocysteine-inserting opal suppressor $tRNA^{Ser}$ (*39*). (H) $tRNA^{Trp}$, position 32 is 2′-*O*-methyl-C (*41*). (I) $tRNA^{Tyr}$ (*42*). (J) Consensus sequence for *E. coli* tRNAs modified by DMAPP−tRNA transferase.

protocol was normally twice the highest final assay concentration. Serial dilutions were carried out after the annealing step. $tRNA^{Phe}$ lacking the i^6A37 modification was heat-annealed by warming the samples to 75 °C followed by gradual cooling to room temperature over a period of 1 h. This slow cooling procedure was also used to form duplexes for the 17-base RNAs. Enzyme was diluted with 50 mM Tris/BSA (1 mg/mL), pH 7.5. Standard assay mixtures (20 μL) contained 50 mM Tris, pH 7.5, 3.5 mM $MgCl_2$, 5 mM BME, 1 mg/mL BSA, 10 μM or 25 μM [1-^{3}H]DMAPP (100 Ci/mol), and varying concentrations of RNA. For minihelix substrates with full activity, the assays generally contained 2.6 nM enzyme, while those with reduced activity required higher concentrations. The samples were prewarmed for 5 min at 37 °C before the reaction was initiated by addition of 5 μL of enzyme solution. Reactions were allowed to run for 5 min at 37 °C and then quenched by addition of 50 μL of 100% ethanol and 2 μL of 3 M sodium acetate, pH 5.2. The samples were allowed to stand overnight at −78 °C to precipitate RNA, and the entire volume was applied to 2.1 cm Whatman GF−C glass fiber filters and allowed to air-dry for 1 h. The filters were washed twice with 10% TCA (10 mL/filter, 10 min each wash), twice with ethanol, and once with diethyl ether, air-dried, and counted by liquid

scintillation spectrometry. Background radioactivity was determined by conducting the assay exactly as described with added buffer instead of enzyme. This value was subtracted from the measured radioactivity for individual points. Kinetic constants were determined as previously described (*22*).

Oligoribonucleotide Melting Curves. RNA was dissolved in a buffer of TE containing 7 mM $MgCl_2$ to a final concentration of 300 μM. The samples were placed in a boiling water bath for 5 min and then cooled rapidly on ice. After 15 min on ice, the samples were diluted to 5 μM, 1.5 μM, and 0.5 μM with 10 mM sodium cacodylate, pH 7.5, containing 3.5 mM $MgCl_2$. Absorbance at 260 nm was monitored for all three concentrations simultaneously on a Beckman DU 740 spectrophotometer as the samples were warmed from 10 to 100 °C at a rate of 0.5 °C/min. Absorbances were measured at 1.0 °C intervals.

Native Polyacrylamide Gel Electrophoresis of RNA Substrates. RNA samples (300 μM) in TE containing 7 mM $MgCl_2$ were placed in boiling water for 5 min and then cooled rapidly on ice to form hairpins or slowly to room temperature over 1 h to form duplexes. After annealing, glycerol was added to the RNA solutions to approximately 25%. The samples were loaded onto a native gel (16 × 20 × 0.1 cm) consisting of 20% acrylamide with a cross-linking ratio of 29:1 acrylamide/bisacrylamide in TBE buffer (50 mM Tris/boric acid and 1 mM EDTA, pH 8.3). Gels were run at 250 V and 4 °C for approximately 6 h after a 2 h preelectrophoresis. RNA was visualized by silver staining (*29*) or by soaking the completed gel in TBE buffer containing 0.4 μg/mL ethidium bromide.

RESULTS

Comparisons of tRNA Sequences. Figure 1 shows the anticodon stem−loop regions of nine *E. coli* tRNAs that are prenylated by DMAPP−tRNA transferase, as well as a consensus sequence. U33 and the A36-A37-A38 motif are completely conserved, as are the G30−C40 and A−U/Ψ or U−A 31−39 base pairs. The only *E. coli* tRNA with the A36-A37-A38 motif that is not modified at A37 with a dimethylallyl unit is $tRNA^{Ser}$ (GGA), which has the nonconserved stem bases G30−Ψ40 and C31−G39 (*30*). The 29−41 base pair is G−C in eight of the sequences and A−U in $tRNA^{Tyr}$, suggesting a preference for a purine−pyrimidine pair at that position. Position 32 is either C or Ψ, and U33 is conserved in all tRNAs. None of the other modified bases, such as pseudouridine, queuine, or 2′-*O*′-methylcytidine, are conserved. Finally, among the *E. coli* tRNAs with a ms^2i^6A modification at position 37, there is no consensus with regard to the length of the variable loop. $tRNA^{Cys}$, $tRNA^{Phe}$, and $tRNA^{Trp}$ have short variable loops of 4 or 5 bases, while $tRNA^{Leu}$, $tRNA^{Ser}$ (CGA), $tRNA^{Ser}$ (UGA), and $tRNA^{Tyr}$ have longer loops of 15, 18, 16, and 13 bases, respectively (*31*).

Steady-State Kinetic Measurements. We measured DMAPP−tRNA transferase activity for nine chemically synthesized 17-base RNA oligoribonucleotide minihelices based on variations in the anticodon stem−loop of unmodified *E. coli* $tRNA^{Phe}$ (Figure 2). Reactions were quenched with ethanol and sodium acetate (see Experimental Procedures) to ensure complete precipitation of the minihelix RNA (*32*). Enzyme concentrations were adjusted so that less than 15% of the limiting substrate was consumed. At low RNA concentrations (2.5 μM) the rate was found to be linear up to 35% conversion and 10 min reaction times. The kinetic parameters k_{cat}, K_m^{RNA}, and k_{cat}/K_m determined for each oligoribonucleotide at saturating concentrations of DMAPP are given in Table 1.

RNA Minihelices as tRNA Substrate Analogues. The 17 base RNA minihelices used in this study were synthesized on a polystyrene support using dimethoxytrityl coupling chemistry. The 2′-hydroxyls of the RNA phosphoramidites were protected by *tert*-butyldimethylsilyl groups. $Minihelix^{Phe}$, which corresponds to the anticodon stem−loop of wild-type unmodified $tRNA^{Phe}$, was a substrate for DMAPP−tRNA transferase. The value for $k_{cat} = 1.01 \pm 0.02\ s^{-1}$ is essentially the same as that of full-length $tRNA^{Phe}$ without the i^6A modification.[3] However, $K_m = 4.7 \pm 0.5$ μM for $minihelix^{Phe}$ is approximately 180 times higher than the K_m for full-length $tRNA^{Phe}$ as measured in pre-steady-state kinetics experiments (*24*).

Variations within the Anticodon Loop. Two oligoribonucleotides were synthesized to evaluate the requirements for A36 and A38 in the anticodon loop. Initial assays on minihelix A36G with 2.6 nM enzyme gave essentially background levels of activity; however, activity was easily detected when the enzyme concentration was increased to 173 nM. Under these conditions $k_{cat}^{A36G} = (3.9 \pm 2) \times 10^{-3}\ s^{-1}$, a 260-fold decrease relative to the reaction with $minihelix^{Phe}$. K_m^{A36G} was 59 ± 8 μM, a 13-fold increase over $minihelix^{Phe}$. The kinetic constants for minihelix A38G were $k_{cat}^{A38G} = 0.111 \pm 0.003\ s^{-1}$ and $K_m^{A38G} = 22 \pm 3$ μM, a 9-fold decrease and a 5-fold increase, respectively.

Variations in the Anticodon Stem. A random coil oligoribonucleotide was synthesized in which the 5-base-pair stem of the helix was completely disrupted by substituting all of the cytidines in the G/C-rich stem with guanosine. At 88 μM RNA and enzyme concentrations up to 432 nM no activity was detected, and it was determined that $k_{cat} < 1 \times 10^{-3}\ s^{-1}$ for this oligoribonucleotide. Minihelix AU31GC, where the A−U base pair at the bottom of the stem was changed to a G−C, had $k_{cat} = 0.67 \pm 0.06\ s^{-1}$, less than a 2-fold decrease from $minihelix^{Phe}$, and $K_m = 12 \pm 3$ μM, less than a 3-fold increase. Minihelix GC30AU, which alters the conserved G−C base pair at position 30−40 in the stem, had $k_{cat} = 0.79 \pm 0.05\ s^{-1}$ and $K_m = 4.5 \pm 1.5$ μM, essentially the same values measured for $minihelix^{Phe}$. Likewise, variant GC30CG gave $k_{cat} = 0.67 \pm 0.02\ s^{-1}$ and $K_m = 6.5 \pm 0.7$ μM, parameters similar to those of $minihelix^{Phe}$. GC29CG/GC30CG, the substrate where both G−C pairs at positions 29−41 and 30−40 were inverted, showed a 4-fold decrease in k_{cat} and a 4-fold increase in K_m.

Structures of the Minihelix Substrates. The RNA substrates used in this study were designed to mimic the anticodon stem−loop region of $tRNA^{Phe}$, which forms a 5-base-pair stem and a 7-base loop, and closely related variants. Potentially $minihelix^{Phe}$ and the A36G and A38G variants could adopt one of several different conformations. For example, MFOLD analysis (*33*) predicts that the thermodynamically most stable conformations for $minihelix^{Phe}$ and

[3] In the study by Moore and Poulter, $k_{cat} = 0.48 \pm 0.01\ s^{-1}$ for $tRNA^{Phe}$ unmodified at A37 was reported. Our experiments with radiolabeled DMAPP from a different source gave a somewhat higher value, $k_{cat} = 1.26 \pm 0.096\ s^{-1}$.

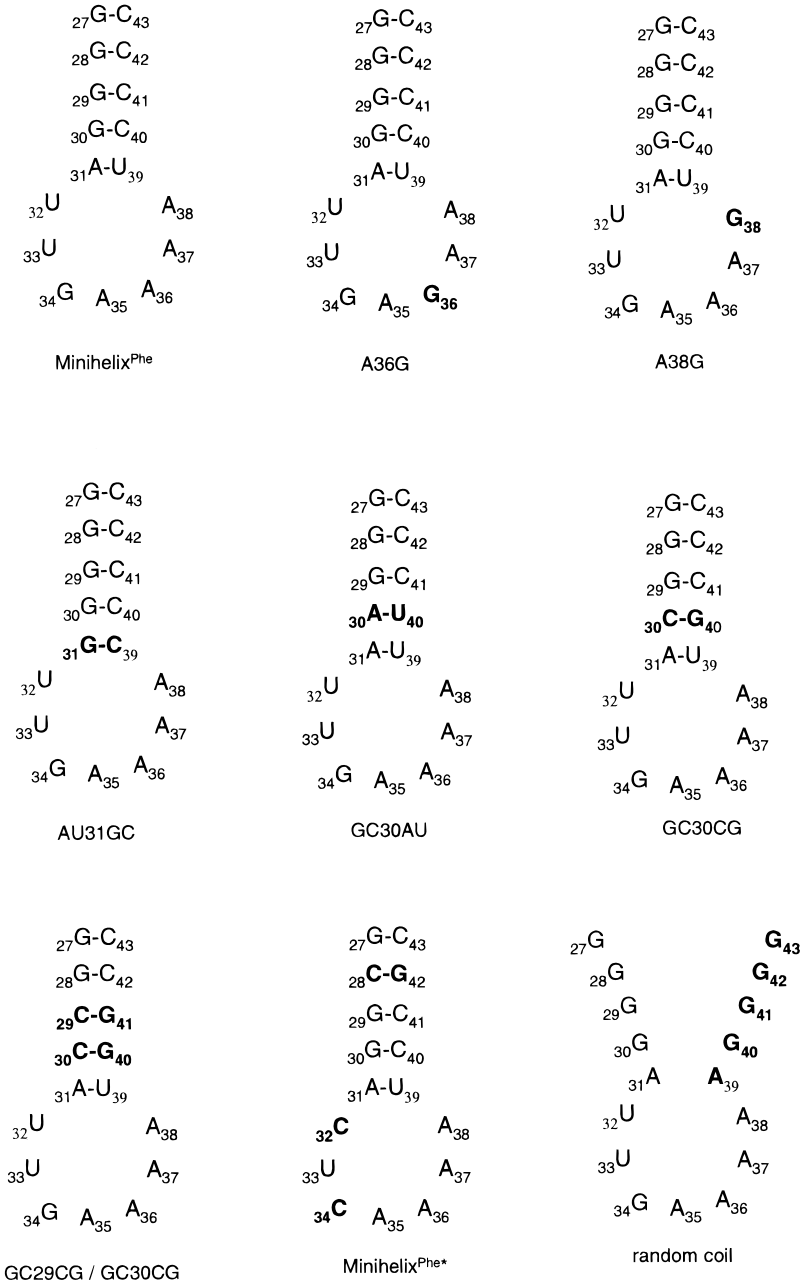


FIGURE 2: Oligoribonucleotides tested as substrates for DMAPP−tRNA transferase.

Table 1: Steady-State Kinetic Constants for Oligoribonucleotide Analogues of the Anticodon Stem−loop Region of *E. Coli* tRNA^{Phe}

substrate	k_{cat} (s^{-1})	K_m (μM)	rel k_{cat}	rel K_m	k_{cat}/K_m (s^{-1} μM^{-1})	rel k_{cat}/K_m
minihelix^{Phe} (hairpin)	1.01 ± 0.02	4.7 ± 0.5	1.0	1.0	0.22 ± 0.02	1.0
minihelix^{Phe} (duplex)	1.03 ± 0.08	3.1 ± 0.7	1.0	0.66	0.33 ± 0.09	1.5
minihelix^{Phe*}	0.75 ± 0.06	3.34 ± 1	0.75	0.72	0.22 ± 0.08	1.0
A36G	$(3.9 \pm 2) \times 10^{-3}$	59 ± 8	3.0×10^{-3}	13	$(6.7 \pm 0.9) \times 10^{-5}$	3.1×10^{-4}
A38G	0.111 ± 0.003	22 ± 3	0.11	4.7	$(5.1 \pm 0.6) \times 10^{-3}$	0.023
AU31G	0.67 ± 0.06	12 ± 3	0.67	2.6	0.055 ± 0.015	0.25
GC30AU	0.79 ± 0.05	4.5 ± 1.5	0.78	0.9	0.18 ± 0.06	0.8
GC30CG	0.67 ± 0.02	6.5 ± 0.7	0.66	1.4	0.10 ± 0.01	0.48
GC29CG/GC30CG	0.26 ± 0.03	19 ± 5	0.26	3.8	0.014 ± 0.004	0.070
random coil	$<1 \times 10^{-3}$					

the A36G variant in 1 M NaCl contain 3-base loops (GAA and GAG, respectively), while the most stable conformation for the variant A38G has a 5-base UGAAA loop closed by a G−U pair. In addition, the four contiguous G−C pairs in the stem of minihelix^{Phe} and related variants could form G-quartet aggregates. Minihelix^{Phe} and the A36G variant could also form a GNRA tetraloop where U33 pairs with A38 and U32 is looped out.

To address concerns about how different conformations might affect the ability of the RNAs to function as substrates, a variant, minihelix^{Phe*}, was prepared for which conformations other than the tRNA-like 5-base-pair stem/7-base loop

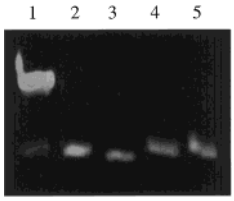


FIGURE 3: Native PAGE of RNA substrates. RNA samples in lanes 2−5 were placed in boiling water for 5 min and then cooled quickly on ice as described under Materials and Methods. Lane 1: minihelix^{Phe}, duplex. Sample was cooled gradually over 1 h to room temperature. Lane 2: minihelix^{Phe}. Lane 3: minihelix^{Phe*}. Lane 4: A36G. Lane 5: A38G.

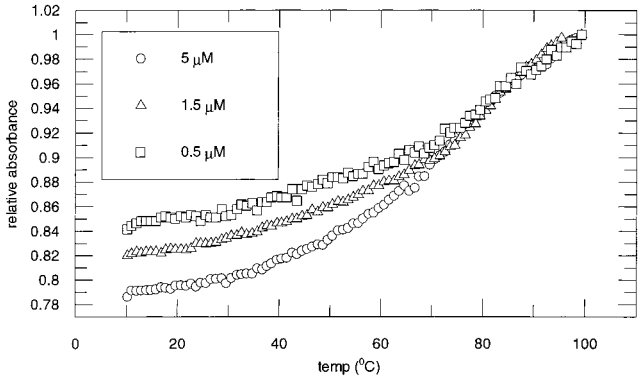


FIGURE 4: Melting curves for minihelix^{Phe} at 5 μM (○), 1.5 μM (△), and 0.5 μM (□). Absorbances at 260 nm are normalized to the 100 °C value for each RNA concentration.

are unlikely. Minihelix^{Phe*} has the following substitutions in the basic minihelix^{Phe} structure: G28−C42 was switched to C28−G42, U32 was changed to C32, and G34 was changed to C34 (Figure 2). Minihelix^{Phe*} was a substrate for the enzyme with kinetic parameters virtually identical to those of minihelix^{Phe}. Thus, any structural differences that might exist between minihelices Phe and Phe* do not substantially alter the ability of the enzyme to recognize the RNA substrates and to catalyze transfer of the dimethylallyl moiety.

Native polyacrylamide gels of the RNA substrates confirmed that the molecules formed stem−loop structures during our rapid annealing protocol. As shown in Figure 3, 20% PAGE of minihelix^{Phe}, minihelix^{Phe*}, A36G, and A38G rapidly annealed on ice at concentrations equal to or slightly higher than those used to prepare samples for enzyme assays gave single bands running slightly above a bromophenol blue standard. However, when minihelix^{Phe} was annealed under conditions favorable for the formation of a duplex structure (high RNA concentration, slow cooling), we observed a heavy band that ran significantly higher on the gel. The random coil 17-base oligoribonucleotide ran between the bands obtained for rapid and slow annealing of minihelix^{Phe} (data not shown). Melting curves were also measured for minihelix^{Phe}, minihelix^{Phe*}, A36G, and A38G in order to demonstrate that the RNA molecules are sufficiently stable to retain their secondary structure during assays at 37 °C (Figure 4). In all cases, the melting curves did not reach a high-temperature plateau by 95 °C. While we were unable to accurately determine a melting temperature for the RNAs, it is evident that the T_ms are in the neighborhood of 80 °C [consistent with an MFOLD analysis (*33*)] and that the samples stably annealed at 37 °C.

The PAGE and melting experiments indicate that the RNA substrates form hairpin structures under rapid annealing conditions. Thus, the reduced catalytic efficiency of the A36G and A38G variants is not due to preferential duplex formation by these RNAs. Significantly, the duplex structure for minihelix^{Phe}, formed by slow annealing at high concentrations, also was a substrate for the enzyme and gave steady-state kinetic parameters similar to those of minihelix^{Phe} hairpin.

DISCUSSION

The structural features important for recognition of tRNAs by their cognate aminoacyl-tRNA synthetases have long been a subject of investigation. The related problem of how tRNA-modifying enzymes recognize specific tRNA substrates from among the pool of all tRNAs is less well studied, but nonetheless intriguing. DMAPP−tRNA transferase catalyzes the transfer of a five-carbon dimethylallyl moiety to the adenosine at position 37 of those tRNAs with flanking adenosines at positions 36 and 38. In this study, we addressed the question of which features in the tRNA substrates are important for recognition by this enzyme. Of specific interest was the extent to which common features of tRNA structure—in particular the stem−loop structure in the anticodon region—are required for recognition. We also wished to determine if a specific sequence motif within the anticodon stem−loop is required for recognition and whether the presence of other modified nucleotides such as pseudouridine and queuine is important. These questions were addressed by measuring the catalytic efficiencies (k_{cat}/K_m) for a series of RNA substrate analogues, where the bases at putative recognition sites suggested by sequence analysis of normal tRNA substrates for *E. coli* DMAPP−tRNA transferase were altered. We focused on local anticodon stem−loop sequence elements, using synthetic 17-base oligoribonucleotides that mimic the anticodon stem−loop region of tRNA.

The lack of a consistent pattern in the length of the variable loops of tRNAs containing the i^{6}A modification leads one to conclude that recognition of the substrate is based primarily on sequence elements near the site of modification, specifically within the anticodon stem-loop. Our results, like those of Leung et al. (*23*), show that minihelix^{Phe}, a 17-base oligoribonucleotide corresponding to the unmodified anticodon stem−loop of *E. coli* tRNA^{Phe}, is sufficient to act as a substrate for DMAPP−tRNA transferase. Modification of minihelix^{Phe} proceeded with a k_{cat} essentially the same as that for full-length undermodified tRNA^{Phe}.

Analysis of the sequences of the tRNAs in *E. coli* that contain the i^{6}A modification strongly suggests that the adenosines flanking A37 are critical for recognition by the enzyme. The A36G minihelix had a k_{cat} only 0.4% that of minihelix^{Phe} and a 13-fold higher K_m , to give a 3000-fold reduction in k_{cat}/K_m. A change in catalytic efficiency of this magnitude indicates that the presence of A36 is required for the prenyl transfer reaction to occur under normal conditions in vivo. Substitution of A38 to G reduced the catalytic efficiency by over 40-fold, indicating that the presence of an adenosine in this position is also highly preferred by the enzyme. The absence of activity above background levels for the "random coil" RNA substrate containing the loop

region of minihelix^{Phe} flanked by noncomplementary sequences demonstrated that the enzyme requires an element of secondary structure in its RNA substrate.

Several putative recognition elements within the stem region suggested by sequence analysis were not in fact required for recognition. The total conservation of a G30-C40 base pair in tRNAs modified with i^{6}A suggests that this motif might be important, while sequence data also suggest that a G−C or C−G base pair at position 31−39 might have a negative influence. This hypothesis is supported by the finding that tRNA^{Ser} (GGA), with the A36-A37-A38 motif but with a G−Ψ mismatch at position 30−40 and a C−G pair at 31−39, is not modified at A37 (*30*). Finally, it might be expected that the complete conservation of G−C or A−U base pairs at position 29−41 reflects a requirement for a purine−pyrimidine base pair at that location. However, none of these proposals were confirmed by our results. The minihelix^{Phe} variant with a G31−C39 pair had nearly the same activity as minihelix^{Phe}. In addition, the completely conserved G30−C40 base pair appears not to be required, as neither inversion to C30−G40 nor a change to A30−U40 significantly affected enzymatic activity. However, simultaneously inverting *both* G29−C41 and G30−C40 to C−G does impart somewhat reduced activity. On the basis of these results, it seems likely that the enzyme does not make critical, base-specific contacts with the helical stem of the tRNA substrate. Rather, changing the composition of the stem by inverting two G−C base pairs to C−G may slightly alter the overall conformation of the helix in this region, possibly disrupting contacts between the protein and the phosphodiester backbone of the RNA substrate.

It is unlikely that the substantially reduced catalytic efficiencies seen for the A36G and A38G variants are due to changes in the conformation of the RNA loop caused by the base substitutions. Minihelix^{Phe}*, minihelix^{Phe}, and the other RNAs we studied with base substitutions outside of the critical A36-A37-A38 region are all good substrates. Furthermore, the PAGE and melting experiments demonstrate that the RNA substrates are hairpins under the annealing conditions used to prepare samples for our assays, eliminating the possibility that the differences in catalytic efficiency observed for A36G and A38G result from the formation of duplex structures. In fact, the duplex of minihelix^{Phe} is an excellent substrate! Motorin et al. (*25*) also identified A36 and A38 as critical bases using full-length tRNA variants. Thus, we conclude that base-specific RNA−protein contacts, rather than conformational effects specific to our 17-base substrate analogues, account for the dramatic changes in catalytic efficiency toward the A36G and A38G substrates.

Finally, it is evident that the presence of other modified nucleosides, such as pseudouridine or queuine, is not essential for substrate recognition. Minihelix^{Phe}, which serves as a substrate with a value of k_{cat} similar to that of full-length tRNA^{Phe}, contained only the four standard RNA bases, as did the other fully active minihelices that we tested. This conclusion is supported by the studies by Leung et al. (*23*) and Moore and Poulter (*22*), in which completely unmodified, in vitro transcribed tRNA^{Phe} (*23*) showed substrate activity similar to that of full-length tRNA^{Phe} unmodified only at position 37 (*22*). Curnow et al. (*32*) have also demonstrated that other modified bases, including ms^{2}i^{6}A, are not required for substrate recognition by tRNA (preQ1) transglycosylase, a key enzyme in the synthesis of queuine at position 34 of some tRNAs. Thus, it appears from these data either that modifications to bases in the anticodon stem−loop region do not occur in any specific order relative to one another or that the i^{6}A and queuine modifications must occur before others in the anticodon stem−loop.

Our results show that DMAPP−tRNA transferase has a fairly broad selectivity with regard to potential tRNA substrates. There is a strong preference for adenosines flanking the site of reaction at A37, but apparently no base-specific recognition motifs exist outside of the flanking adenosines. In this respect, DMAPP−tRNA transferase is similar to tRNA (preQ1) transglycosylase, which has also been shown to accept a 17-base RNA minihelix as a substrate. For this enzyme, recognition requirements were limited to the presence of an intact helical stem and a 7-base loop containing a U33-G34-U35 motif (*34*). All that appears to be absolutely required for substrate recognition and catalysis by DMAPP−tRNA transferase is an intact double-stranded helical region adjacent to a loop containing the A36-A37-A38 motif. Even the duplex form of minihelix^{Phe} is an efficient substrate for the enzyme.

The observation that minihelix^{Phe} serves as a substrate for DMAPP−tRNA transferase, but with a value for K_m 2 orders of magnitude higher than the natural substrate (in these experiments full-length tRNA^{Phe} unmodified at position 37), suggests that the enzyme makes contacts with the tRNA substrate outside of the anticodon region that enhance substrate binding. However, the fact that the enzyme catalyzes modification of the minihelix and full-length tRNA with the same value for k_{cat} suggests that these contacts are distant from the active site and do not affect the chemical step.

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