Stereochemistry of tRNA(m^{5}U54)-Methyltransferase Catalysis: ^{19}F NMR Spectroscopy of an Enzyme−FUraRNA Covalent Complex†

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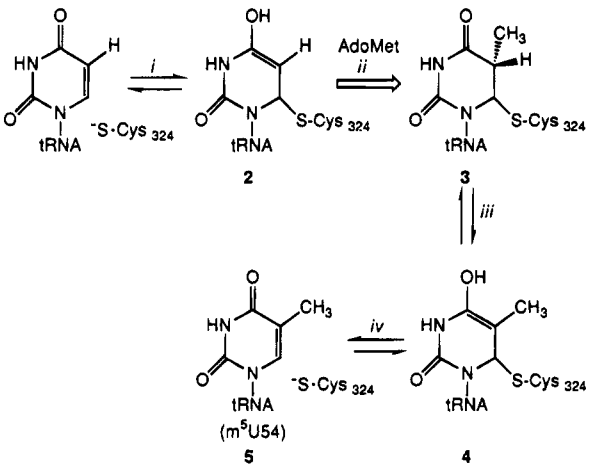
ABSTRACT: The catalytic mechanism of tRNA(m^{5}U54)-methyltransferase (RUMT) involves the formation of a covalent adduct between Cys324 of RUMT and C6 of Ura54 in tRNA. The covalent adduct is subsequently methylated at C5 by *S*-adenosyl-L-methionine (AdoMet). We used an RNA substrate analog containing 5-fluorouracil (FUra) in place of Ura54 to trap the covalent complex and analyzed the adduct by ^{19}F NMR spectroscopy. The ^{19}F NMR spectrum of the adduct consisted of an overlapping doublet of quartets, with an H_6-F coupling constant of 4 Hz and a CH_3-F coupling constant of 22.4 Hz. On the basis of the magnitude of the H_6-F coupling constant, we determined that Cys324 of RUMT and the methyl moiety from AdoMet added across the 5,6-double bond of FUra54 in *cis* fashion. We deduced that the nucleophilic addition was also *cis* in the normal enzymatic reaction and that the subsequent β-elimination of the 5-H and catalytic cysteine was *trans*. Further, on the basis of chemical considerations, we proposed several conformational adaptations of enzyme−substrate complexes that must occur on the reaction pathway. Together with previous studies, this study enables the proposal of the complete stereochemical pathway for the RUMT-catalyzed methylation of Ura54 in tRNA.

tRNA(m^{5}U54)-methyltransferase (RUMT,[1] EC 2.1.1.35) catalyzes the AdoMet-dependent methylation of Ura54 in the TΨC-loop of all *Escherichia coli* tRNAs. The enzyme also catalyzes the methylation of small ribonucleotide oligomers corresponding to the T-arm of tRNA (Gu & Santi, 1991). The mechanism of RUMT-catalyzed methylation of tRNA is fairly well understood and is analogous to that elucidated for the enzymes thymidylate synthase and DNA-cytosine methyltransferase [for reviews, see Santi and Danenberg (1984) and Ivanetich and Santi (1992)]. As shown in Scheme 1, the reaction occurs by (i) nucleophilic addition of Cys324 of RUMT to the 6-carbon of Ura54 in RNA to give a covalent binary complex, **2** (Kealey & Santi, 1991; Gu & Santi, 1992), followed by (ii) direct methyl transfer (probably by S_N2 displacement) from AdoMet to the 5-position of Ura54 to give the methylated covalent complex, **3** (Kealey et al., 1991), (iii) abstraction of the C5 proton, and (iv) β-elimination of the enzyme to yield m^{5}Ura54.

Although many aspects of the mechanism are understood, stereochemical details are lacking. The Michael adduct **3** forms by either *cis* or *trans* addition of the Cys and methyl group across the C5−C6 double bond; the subsequent β-elimination of the 5-H and enzyme must occur with the opposite stereochemistry to yield products. A knowledge of the cryptic stereochemistry of this reaction would provide details of the reaction pathway necessary for a complete understanding of the enzymatic mechanism.

Scheme 1



The stereochemistry of the analogous TS and DCMT reactions have been elucidated by structural studies of the stable covalent adducts formed with enzyme, cofactor, and nucleotide containing a 5-fluorinated pyrimidine (James et al., 1976; Byrd et al., 1978; Matthews et al., 1990b; Klimasauskas et al., 1994). For both of these enzymes, the addition reaction was shown to be *trans*, and the elimination reaction was deduced to be *cis*. Similar to these systems, when 5-FUra is substituted for Ura54 in tRNA, a methylated covalent complex analogous to the steady state intermediate **3** is formed that contains a fluorine at C5 instead of hydrogen (Scheme 2, **7**) (Santi & Hardy, 1987). This inhibitory

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[1] Abbreviations: RUMT, tRNA(m^{5}U54)-methyltransferase; tRNA-(m^{5}U54), 5-methyluridine at position 54 of tRNA; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; FUraRNA, tRNA or T-arm of tRNA containing 5-fluorouridine in place of uridine at position 54; FUratRNA, tRNA with the substitution of 5-fluorouridine for all uridines; dFUraT-arm, T-arm analog in which Urd54 is replaced with 5-fluoro-2′-deoxyuridine (see Figure 1); RUMT−FUraRNA-CH_3, covalent complex containing RUMT, FUraRNA, and the methyl derived from AdoMet; TS, thymidylate synthase; DCMT, DNA−cytosine methyltransferase; TS−FdUMP−CH_2H_4folate, covalent complex consisting of TS, 5,10-methylene-5,6,7,8-tetrahydrofolate, and 5-fluoro-2′-deoxyuridine 5′-monophosphate; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; FID, free induction decay.

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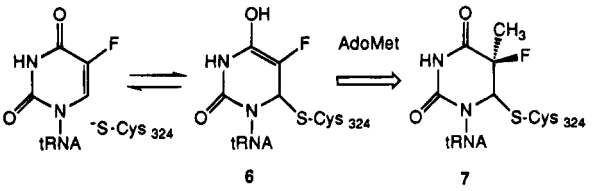
Scheme 2



complex is sufficiently stable to allow direct study and has been valuable in elucidating the mechanistic details of this enzyme (Kealey & Santi, 1991; Gu & Santi, 1992).

In this study, we employ ^{19}F NMR spectroscopy to elucidate the relative stereochemistry of the addition of Cys324 of RUMT and the methyl moiety of AdoMet to an oligonucleotide T-arm analog containing FUra. The magnitude of the H_6-F coupling constant of the adduct was used to distinguish between isomers in which H_6 and F are *trans* and those in which H_6 and F are *cis*. The results show that, in contrast to TS and DCMT, RUMT proceeds by *cis* addition, followed by *trans* elimination.

MATERIALS AND METHODS

RUMT was prepared as described previously (Kealey & Santi, 1994). All RNA phosphoramidites and derivatized CPG column supports were purchased from Chemgenes Corporation (Waltham, MA). Reagents for solid phase RNA synthesis were obtained from Applied Biosystems Inc. (Foster City, CA). Tetra-*n*-butylammonium fluoride (TBAF)/THF was obtained from Aldrich. DE-52 was purchased from Whatman. α-Chymotrypsin was obtained from Worthington. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (71 Ci/mmol) was purchased from Amersham. Methylation buffer consisted of 50 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, and 1 mM DTT.

Preparation of RNA. The T-arm analog, 5′-GUGUG(5-fluoro-2′-deoxyU)UCGAUCCACAC-3′ (Figure 1), was prepared by solid phase synthesis as follows: DMT ribocytidine succinyl aminopropyl CPG resin (0.43 g, 6.5 μmol C) was loosely packed into a 0.7 × 5 cm column, and RNA synthesis was carried out on an Applied Biosystems 394 oligonucleotide synthesizer, according to instructions provided by Applied Biosystems. A second round of RNA synthesis was performed as described earlier, using the remainder of the resin (ca. 0.3 g). Following synthesis and cleavage from the solid support, base protecting groups were removed by incubating the RNA in 70% NH_4OH/30% ethanol (v/v) for 12 h at 55 °C. The ammonia was removed by evaporation with a steady stream of air, and the RNA was dried *in vacuo*. The RNA was dissolved in 1 M TBAF/THF (1 mL/μmol of immobilized cytidine) and incubated at 25 °C in the dark for 5 h. Complete silyl deprotection was verified by C-4 reverse phase HPLC (Webster et al., 1991). The THF was removed by evaporation, and 3 vol of 0.3 M ammonium acetate (pH 7.0) was added to the resulting viscous solution. The RNA was desalted by dilution with ammonium acetate, followed by concentration in an Amicon Centriprep 3 concentrator. Tightly bound TBA ions were removed by adsorbing the RNA (3.8 mL) to a 10 mL DE-52 column (2.5 × 10 cm), equilibrated with 20 mM Tris-HCl and 50 mM NaCl (pH 8). The column was washed with 45 mL of equilibration buffer, and the RNA was eluted in equilibration buffer containing 0.5 M NaCl. The RNA (13 mL) was concentrated 6-fold in an Amicon Centriprep 3 concentrator and then precipitated by the addition of 2.5 vol of cold 95% ethanol. Approximately 12 OD RNA was recovered per micromole of immobilized cytidine.

The T-arm analog containing dUra54 in place of Ura54 was synthesized on the 1 μmol scale, as described earlier, and the 2′-deprotected dUra54 T-arm was purified by C-4 reverse phase HPLC (Webster et al., 1991). The ability of the dUraT-arm to accept methyl groups from AdoMet, catalyzed by RUMT, was assessed in an in vitro methylation assay. The dUraT-arm (21 μM) was incubated at 15 °C in 28 μL of methylation buffer containing [methyl-^{3}H]AdoMet (47 μM, 1 Ci/mmol) and RUMT (3 μM). After 10 min, the reactions were stopped, aliquots were applied to DE-81 filters, and the incorporation of ^{3}H-labeled methyl groups into the RNA was quantitated as described (Gu & Santi, 1992).

Formation and Protease and RNAase Digestion of the RUMT−dFUraT-arm-CH_3 Covalent Complex. A reaction mixture (11.9 mL in methylation buffer) containing RUMT (16 mg), dFUraT-arm (110 OD), and [*methyl*-^{3}H]AdoMet (1.79 μmol, 0.055 Ci/mmol) was incubated at room temperature, and the progress of the reaction was monitored by reverse phase HPLC of the free RNA. When all of the RNA had reacted (21 h), the sample was clarified by centrifugation, diluted 3.5-fold with D_2O, and concentrated to 0.5 mL in an Amicon Centriprep 30 concentrator. Formation of the covalent complex was verified by 12% SDS−PAGE, with Coomassie and ethidium bromide staining, and by ^{19}F NMR (see the following).

To the RUMT−dFUraT-arm-CH_3 covalent complex (0.5 mL) was added solid urea to a final concentration of 2 M, followed by 20 μL of α-chymotrypsin (200 μg). After 14 h of incubation at 37 °C, a second aliquot (200 μg) of α-chymotrypsin was added, and the sample was incubated for an additional 8 h at 37 °C. Upon digestion, a precipitate formed that contained 25% of the total RUMT−dFUraT-arm-CH_3 complex. The precipitate was dissolved in 400 μL of 8 M urea and diluted to 4 M urea with water, and another 200 μg of α-chymotrypsin was added. The sample was incubated at 37 °C for 12 h and then clarified by centrifugation, and the supernatant was desalted and concentrated to 400 μL in an Amicon Centricon 3 microconcentrator. To the concentrated chymotrypsin digest (400 μL) was added 100 μg of RNase A (10 mg/mL), and the sample was incubated overnight at 37 °C. Digestions of the covalent complex were monitored by SDS−PAGE (detection by Coomassie staining), reverse phase HPLC (detection by UV absorption and scintillation counting of ^{3}H in HPLC fractions), and ^{19}F NMR spectroscopy.

^{19}F NMR Spectroscopy. ^{19}F NMR spectra were obtained at 282 MHz (25 °C) on a GE QE-300 pulsed FT NMR spectrometer, fitted with a 5 mm fluorine probe. For most samples, data were accumulated by using 16 000 data points, a 9 μs pulse width, a 1 s relaxation delay, and a spectral width of 12 000 Hz. For the final spectrum of the nuclease- and protease-digested covalent complex, a spectral width of 5681 Hz was employed. For each NMR experiment, about 16 000 scans were collected and averaged. Prior to Fourier transformation, the FID was apodized by exponential multiplication, using a line broadening of 4 Hz. Theoretical spectra were calculated by the program CHRISM (QE-Charm software package).

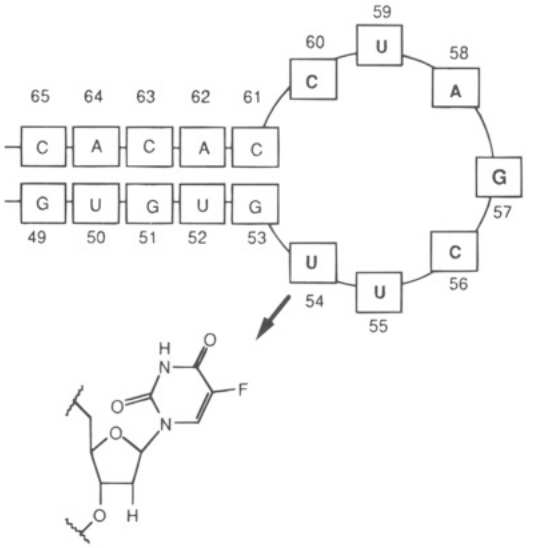


FIGURE 1: Structure of the T-arm analog of tRNA used to form the RUMT−dFUraTarm-CH_3 covalent complex.

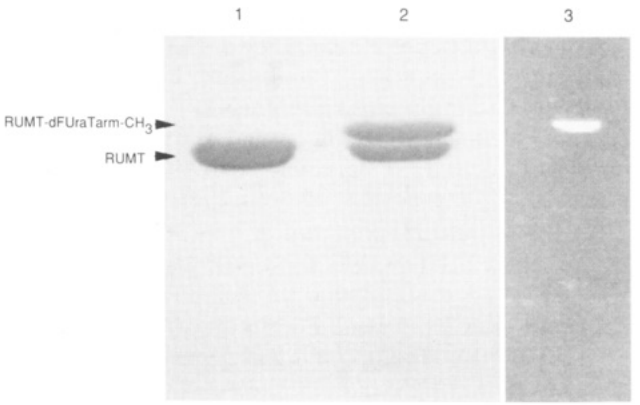


FIGURE 2: SDS−PAGE of the RUMT−dFUraTarm-CH_3 covalent complex: lane 1, RUMT stained with Coomassie R-250; lane 2, RUMT−dFUraT-arm-CH_3 stained with Coomassie R-250; lane 3, RUMT−dFUraT-arm-CH_3 stained with ethidium bromide.

RESULTS

The RNA substrate used in this study was the 17-mer dFUraT-arm analog shown in Figure 1. The T-arm rather than full-length tRNA was employed to easily allow placement of a unique 5-FUra at position 54 by chemical synthesis; the 5-fluorodeoxyribose analog was used because it was commercially available. Solid phase RNA synthesis yielded 120 OD (ca. 1 μmol) dFUraT-arm that, following DEAE chromatography and desalting, was 70−80% pure, as estimated by denaturing PAGE. Prior to large-scale synthesis of the dFUraT-arm, the corresponding dUraT-arm was synthesized and assayed for RUMT-catalyzed methylation by AdoMet. The initial velocity of methylation of the dUraT-arm was 5-fold slower than that of wild-type (wt) T-arm, and the reaction proceeded to completion.

Treatment of limiting dFUra54T-arm with excess RUMT and AdoMet resulted in >95% conversion of the RNA to the RUMT−dFUraT-arm-CH_3 complex. Formation of the RUMT−dFUraT-arm-CH_3 covalent complex was accompanied by the disappearance of the free RNA peak on HPLC and the appearance of a new band on SDS−PAGE (Figure 2); the new band stained with both Coomassie R-250 and ethidium bromide, verifying that it contained protein and nucleic acid. ^{19}F NMR of the complex revealed the

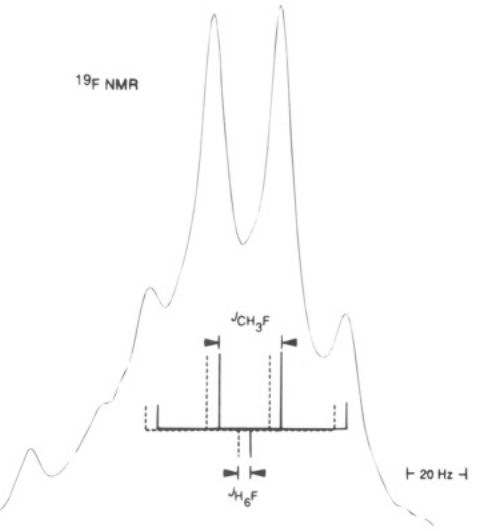


FIGURE 3: ^{19}F NMR spectrum of the peptide−dFUra(nucleotide)-CH_3 complex, showing the proposed coupling constants. One of the quartets is represented by dashed lines to emphasize that the quartets overlap.

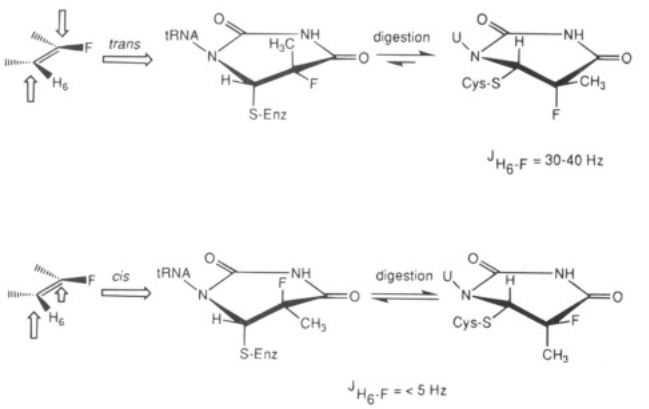


FIGURE 4: Stereochemistry of nucleophilic addition. Upon denaturation, the stable form of the *trans* isomer (path a) is that in which the fluorine and H_6 are *trans* diaxial; in *cis* addition (path b), there is no conformational isomer that directs the fluorine and H_6 *trans* diaxial.

disappearance of the ^{19}F resonance associated with free T-arm and the appearance of a new, broader resonance characteristic of a large macromolecular complex (Byrd et al., 1978).

Because of the severe line broadening of the fluorine signal in the complex, it was necessary to reduce the molecular mass of the complex. *In situ* digestion of the RUMT−dFUra-arm-CH_3 complex with chymotrypsin and RNAase A yielded a peptide−dFUra(nucleotide)-CH_3 complex. Digestion of the complex was monitored by SDS−PAGE, which verified the complete disappearance of the band associated with the intact complex.

The ^{19}F spectrum of the chymotrypsin-digested adduct consisted of a broad multiplet, which, following RNAse digestion, resolved into an overlapping doublet of quartets, with a $J_{H_6\text{-}F}$ of 4 Hz and a $J_{CH_3\text{-}F}$ of 22.4 Hz (Figure 3). The theoretical ^{19}F NMR spectrum of the adduct consists of a doublet of quartets, due to splitting of the ^{19}F signal by H_5 ($J_{H_6\text{-}F}$) and the C5 methyl protons ($J_{CH_3\text{-}F}$). The splitting pattern of the ^{19}F resonance of the peptide−dFUra(nucleotide)-CH_3 complex thus is consistent with the proposed structure of the covalent adduct, **7** (Santi & Hardy, 1987; Kealey & Santi, 1991).

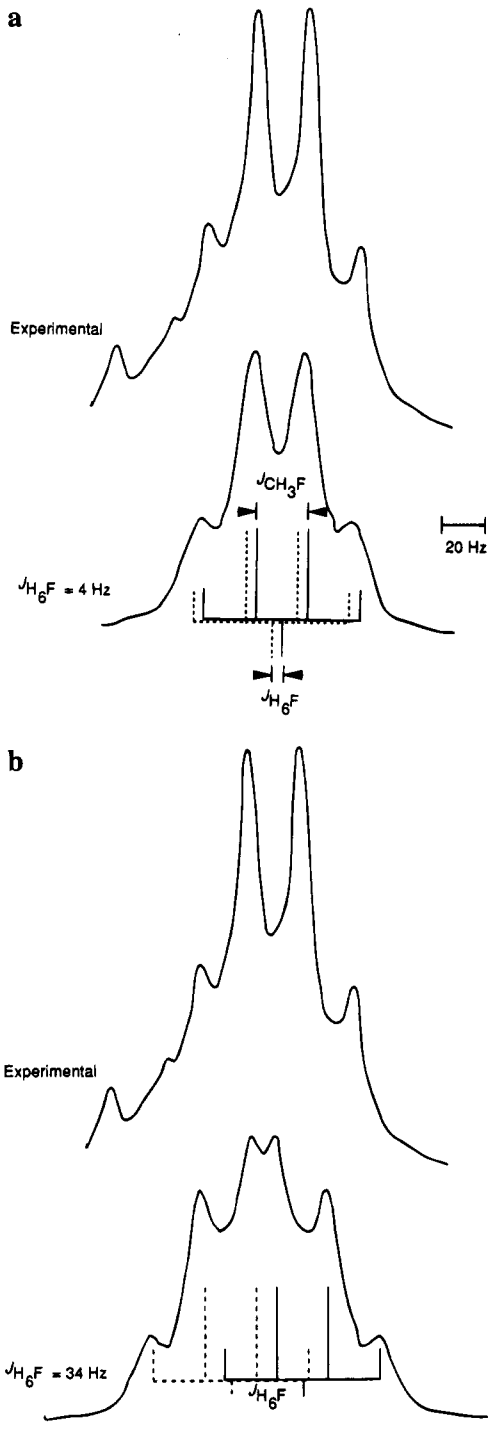


FIGURE 5: (a) ^{19}F NMR spectrum of the peptide−dFU(nucleotide)-CH_3 complex with the theoretical spectrum calculated by the program CHRISM underneath (QE-Charm software package), using experimentally determined coupling constants ($J_{H_6\text{-}F}$ = 4 Hz, $J_{CH_3\text{-}F}$ = 22.4 Hz) and a line width of 15 Hz. (b) Same as (a), except that the bottom spectrum is the theoretical spectrum for a $J_{H_6\text{-}F}$ of 34 Hz.

In addition to confirming the covalent nature of the peptide−dFUra(nucleotide)-CH_3 complex **7**, the ^{19}F NMR spectrum of the adduct contains stereochemical information. As discussed in the following, the magnitude of $J_{H_6\text{-}F}$ depends on the dihedral angle between H_6 and F. A minimum value of $J_{H_6\text{-}F}$ (<5 Hz) is indicative of an H_6-F dihedral angle of about 60°, whereas a maximum value of $J_{H_6\text{-}F}$ (30−40 Hz) indicates a dihedral angle of about 180° (Byrd et al., 1978). We calculated the theoretical spectra for the peptide−dFUra(nucleotide)-CH_3 adduct, using $J_{CH_3\text{-}F}$ of 22.4 Hz (measured), a line width of 15 Hz, and a range of values for $J_{H_6\text{-}F}$ (from 4 to 40 Hz). When $J_{H_6\text{-}F}$ was the minimum value (4 Hz), the theoretical spectrum most closely resembled the experimental spectrum (Figure 5a). Figure 5b shows the theoretical spectrum for a $J_{H_6\text{-}F}$ of 34 Hz (close to the maximum value) and a $J_{CH_3\text{-}F}$ of 22.4 Hz. In this case, the theoretical spectrum does not match the experimentally obtained spectrum of the peptide−dFUra(nucleotide)-CH_3 adduct.

In the methylation reaction catalyzed by TS, the TS−FdUMP−CH_2H_4folate adduct arises by *trans* addition of the catalytic nucleophile and the 1-carbon unit. In this case, $J_{H_6\text{-}F}$ is 34 Hz and $J_{CH_2\text{-}F}$ is 19.5 Hz (James et al., 1976). Hence, although the magnitudes of $J_{CH_{(2,3)}\text{-}F}$ are similar for both the RUMT and TS complexes, the magnitudes of $J_{H_6\text{-}F}$ differ considerably: in the peptide−FdUMP−CH_2H_4folate complex, $J_{H_6\text{-}F}$ approaches a maximum value (34 Hz), whereas in the peptide−dFUra(nucleotide)-CH_3 complex, $J_{H_6\text{-}F}$ approaches a minimum value (4 Hz).

DISCUSSION

Using ^{19}F NMR spectroscopy, we determined the stereochemistry of the covalent RUMT−FUraRNA-CH_3 adduct. From this, we deduced the stereochemical course of the addition−elimination reaction, including several conformational adaptations the complex must make during the RUMT-catalyzed methylation of Ura54 of tRNA.

The basic approach used has been previously described in ^{19}F NMR studies of the TS−FdUMP−CH_2H_4folate complex (James et al., 1976; Byrd et al., 1978) and is briefly outlined here. The ^{19}F NMR spectrum of the adduct is expected to consist of a doublet of quartets, due to scalar coupling to H_6 ($J_{H_6\text{-}F}$) and to the three C5 methyl protons ($J_{CH_3\text{-}F}$). As is typical for vicinal coupling constants, $J_{H_6\text{-}F}$ depends on the dihedral angle between H_6 and F and is described by the empirical Karplus equation. Byrd et al. (1978) determined a Karplus relationship for the C6−H6 and C5−F bonds of the TS−FdUMP−CH_2H_4folate adduct on the basis of parameters obtained from the crystal structure of the model, 1-methyl-5-fluoro-6-methoxy-5,6-dihydrouracil (James & Matsushima, 1976). A maximum value of $J_{H_6\text{-}F}$ (ca. 35 Hz) was obtained when the H_6-F dihedral angle was 180° (diaxial), whereas a $J_{H_6\text{-}F}$ of 4 Hz corresponded to a dihedral angle of 60°. Due to the trigonal geometry of the C2 and C4 carbonyl groups, 1-methyl-5-fluoro-6-methoxy-5,6-dihydrouracil does not adopt a true chair conformation; however, the C5 and C6 substituents are staggered, as observed in substituted cyclohexane (James & Matsushima, 1976). Moreover, the crystal structure of the TS−FdUMP−CH_2H_4folate complex revealed that the C5 and C6 substituents are staggered, with a dihedral angle of 178° for the peptide and CH_2H_4folate groups (Matthews et al., 1990b). We therefore expect the C5 and C6 substituents of the peptide−dFUra(nucleotide)-CH_3 adduct to be staggered, and we refer to their geometries as axial and equatorial, rather than pseudoaxial and pseudoequatorial.

In the *cis* adduct there is no conformation that places H_6 and F in a diaxial arrangement; either H_6 is axial and F is equatorial, or H_6 is equatorial and F is axial (Figure 4, path b). In either case, the H_6-F dihedral angle will be 60° and a minimum $J_{H_6\text{-}F}$ will be observed. In the thermodynamically stable form of the *trans* adduct, the large substituents at C6

(peptide) and C5 (methyl) would assume a diequatorial arrangement; the H and F substituents would be forced into a diaxial arrangement with a dihedral angle of 180°, and a maximum $J_{H_6\text{-}F}$ would be expected [see James et al. (1976)] (Figure 4, path a). Thus, the magnitude of $J_{H_6\text{-}F}$ can be used to distinguish *cis* from *trans* addition. It should be emphasized that this analysis depends on the assumption that, in the denatured or protease-/nuclease-digested complex, bulky substituents will be directed to equatorial positions. Indeed, this was shown to be the case in the TS−FdUMP−CH_2H_4-folate complex, in which the enzyme stabilizes a high-energy *trans* diaxial conformation of the bulky C5 and C6 substituents ($J_{H_6\text{-}F} \leq 4$ Hz), which relaxes to the more stable *trans* diequatorial form upon denaturation ($J_{H_6\text{-}F}$ of ca. 34 Hz) (James et al., 1976; Byrd et al., 1978).

Preliminary attempts utilizing *in vivo* synthesized FUratRNA (ca. 17 FUra/tRNA) for NMR studies were unsuccessful. It was not possible to remove all extraneous FUra-containing nucleotides by RNAse digestion, which complicated spectral assignment. We therefore sought to design a smaller FUraRNA containing only a single FUra, which would simplify spectral assignment and obviate the need for extensive purification of the adduct. The 17-nucleotide T-arm of tRNA and the analog with dU at position 54 are adequate substrates for RUMT. dFUra was specifically incorporated into the target site of the 17-mer T-arm by chemical synthesis for use in the NMR experiments. Using excess RUMT and AdoMet, all of the dFUraT-arm was converted to the RUMT−dFUraT-arm-CH_3 complex. The molecular mass of the covalent complex was reduced by *in situ* digestion with protease and nuclease. The effect of this was 2-fold. First, it reduced line broadening to ca. 10 Hz and enabled the observation of coupling constants larger than 5 Hz. Second, it denatured the system to its thermodynamically stable form.

The ^{19}F NMR spectrum of the peptide−dFUra(nucleotide)-CH_3 complex (Figure 4) consists of a doublet of quartets, with a minimum coupling constant, $J_{H_6\text{-}F}$, of about 4 Hz. On the basis of the aforementioned Karplus equation for the C6−H6 and C5−F bonds of 1-methyl-5-fluoro-6-methoxy-5,6-dihydrouracil [see Byrd et al. (1978)] and coupling constants of model compounds (Gutowsky et al., 1962; Bovey et al., 1964), the minimum $J_{H_6\text{-}F}$ obtained here suggests that H_6 and F are *gauche*, as shown in Figure 4 (path b). This structure could only have arisen by *cis* addition of the catalytic nucleophile and methyl electrophile. It is reasonable to conclude that addition to the normal substrate is also *cis*.

Having established that the addition is *cis*, we deduced that the subsequent elimination of 5-H and enzyme must be *trans*. Further, the chemistry of these processes suggests that small, albeit important, conformational changes in covalent intermediates must occur during catalysis. A group reacting with the π-system of the uracil heterocycle must approach approximately orthogonal to the plane of the ring (Figure 6). By microscopic reversibility, a similar orientation is required when a group departs to reform the π-system. Thus, the initial approach of the thiolate of Cys324 to the electrophilic 6-carbon of Ura54 should be perpendicular to the plane of the heterocycle. The approach of the methyl group from AdoMet should also be perpendicular to the plane of the ring and, based on data presented here, *cis* to the nucleophile attached to the 6-position. Consequently, in **10**, the 5-methyl group would exist in an axial position and the

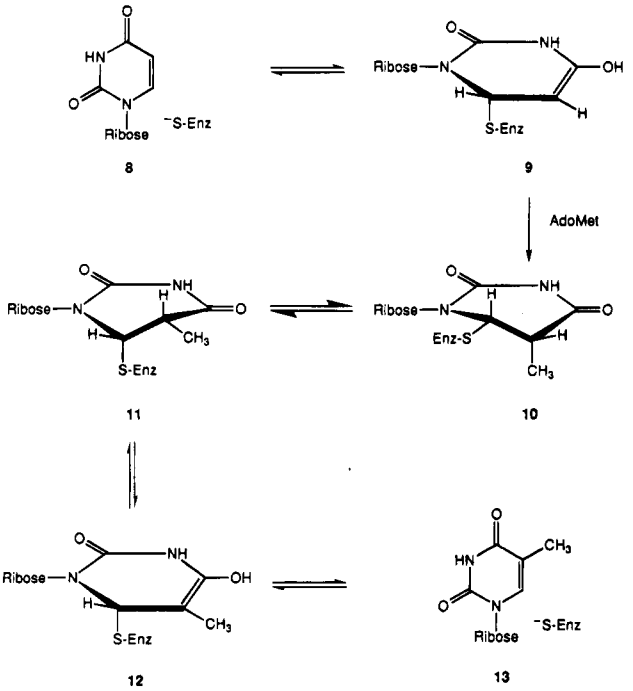


FIGURE 6: Proposed catalytic mechanism of tRNA(m^{5}U54)-methyltransferase showing the stereochemistry of addition. Cys324 is depicted as adding to the *si* face, but the absolute stereochemistry of the addition product, as shown, is arbitrary.

5-hydrogen would be equatorial. For the subsequent elimination reaction, the proton from the 5-position must be in the axial position, as in intermediate **11**, prior to its abstraction to form the enol, **12**. A conformational change in the pyrimidine ring thus is required after addition of the methyl moiety but before abstraction of the 5-proton, resulting in inversion of the 5- and 6-positions of the nucleotide intermediates (i.e., **10** ↔ **11**).

To date, the stereochemistry of nucleophilic addition has been determined for TS and DCMT, both of which are mechanistically similar to RUMT. TS catalyzes the CH_2H_4-folate-dependent methylation of dUMP. Both NMR and crystallographic studies have shown that the catalytic nucleophile of TS and the methylene electrophile add *trans* across C5−C6 of the pyrimidine (James et al., 1976; Matthews et al., 1990a; Montfort et al., 1990). *Hha*I methylase catalyzes the AdoMet-dependent methylation of a specific Cyd within a DNA double helix (Wu & Santi, 1987). Unlike TS, which methylates a mononucleotide, *Hha*I methylates a Cyd within a DNA double helix. It was recently shown by X-ray analysis that *Hha*I "flips" the target Cyd out of the double helix. *Hha*I thus can easily access C5 and C6 from above and below the plane of the pyrimidine, and addition of the catalytic nucleophile and methyl electrophile occurs *trans* (Klimasauskas et al., 1994).

A question that remains is how RUMT gains access to Ura54 of tRNA. From the 3-dimensional structure of tRNA, it is clear that the T-arm itself must first be released from interactions with other regions of tRNA in order for RUMT to facilitate the chemistry we have described earlier. Work in progress suggests that RUMT plays an active role in releasing constraints by the D-loop on the T-arm (X. Gu and D. V. Santi, unpublished observation). Further, in the T-arm of tRNA, Ura54 is stacked between G53 and U55, and C5 and C6 of Ura54 are almost completely buried. At least one face of Ura54 must be extensively exposed to allow access

of the enzyme and AdoMet to the C6 and C5 positions. Whether the T-arm has a floppy loop, which permits access, or whether RUMT induces conformational changes analogous to those induced by *Hha*I methylase (Klimasauskas et al., 1994) remains to be resolved. To address such questions, a crystal structure of RUMT is clearly desirable. Particularly enlightening would be a structure of the RUMT−FUraRNA-CH_3 complex, and crystallographic studies of this complex are in progress.

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