Covalent Adducts between tRNA (m^5U54)-Methyltransferase and RNA Substrates†

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ABSTRACT: The interaction of tRNA (m^5U54)-methyltransferase (RUMT) with in vitro synthesized unmodified tRNA and a 17-base oligoribonucleotide analog of the T-arm of tRNA in the absence of AdoMet has been investigated. Binary complexes are formed which are isolable on nitrocellulose filters and are composed of noncovalent and covalent complexes in nearly equal amounts. The covalent RUMT–RNA complexes are stable to SDS–PAGE and migrate slower than free enzyme or RNA. Kinetic and thermodynamic constants involved in formation and disruption of noncovalent and covalent binary complexes have been determined and interpreted in the context of steady-state kinetic parameters of the enzyme-catalyzed methylation and 5-H exchange of substrate. The results show that the isolable covalent complex is kinetically incompetent as an intermediate for methylation. Isotope trapping experiments show that when AdoMet is added to preformed binary complex, all bound tRNA is converted to methylated product; thus, the covalent complexes are chemically competent to form products. We have concluded that, after a reversible binary complex is formed, the catalytic thiol adds to the 6-carbon of the U54 of tRNA. The initial adduct leaves the reaction pathway to protonation at carbon 5; the latter can deprotonate and re-enter the pathway to form methylated product. It is speculated that covalent binary RUMT–RNA adducts may serve as depots of enzyme–tRNA complexes primed for methylation, or in unknown roles with RNAs other than tRNA.

tRNA (m^5U54)-methyltransferase (RUMT;[1] EC 2.1.1.35), the *trmA* gene product, catalyzes the transfer of the methyl group of AdoMet to U54 of most tRNAs. Regardless of its ubiquitous presence, m^5U at position 54 of tRNA is not essential for growth and survival, and the biological role of RUMT remains uncertain (Bjork, 1992). Recently, we have shown that RUMT catalyzes methylation of a 17-base oligoribonucleotide corresponding to the T-arm of tRNA and suggested that the enzyme might recognize other RNAs which have a minimal consensus sequence (Gu & Santi, 1991b).

The catalytic mechanism of RUMT is analogous to thymidylate synthase and the DNA–(m^5C)-methyltransferases (Santi & Danenberg, 1984; Santi & Hardy, 1987; Wu & Santi, 1987; Chen et al., 1991). The mechanism involves initial formation of a covalent Michael adduct between the thiol of Cys 324 and the 6-carbon of U54 of tRNA (Santi & Hardy, 1987; Kealey & Santi, 1991) which serves to activate the 5-position of U54 for subsequent one-carbon transfer. We have previously reported that, in the presence of AdoMet, RUMT and FUra-tRNA form a methylated covalent complex which is an analog of a steady-state intermediate and can be monitored by a mobility decrease of RUMT on SDS–PAGE (Santi & Hardy, 1987).

In the present work, we show that, in the absence of AdoMet, RUMT forms binary covalent complexes with unmodified substrate tRNA or a 17mer analog of the tRNA T-arm. Unlike the methylated FUra-tRNA–RUMT complexes, the binary RUMT–substrate complexes are reversible. Kinetic and thermodynamic analyses of the binary complexes have been performed, and results have been interpreted to characterize these complexes and assess their relevance in catalysis. Finally, we have proposed that binary covalent complexes between RUMT and RNA may be important in roles other than methylation of tRNA.

MATERIALS AND METHODS

FUra-$tRNA^{Val}$ was a gift from J. Horowitz (Department of Biochemistry and Biophysics, Iowa State University), and plasmid p67YF0 used for preparation of yeast $tRNA^{Phe}$ was a gift from O. C. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado). Yeast $tRNA^{Phe}$ was from Boehringer Mannheim. T4 RNA ligase was from New England Biolabs. T7 RNA polymerase was isolated from *Escherichia coli* BL21 harboring the plasmid pAR1219 (J. J. Dann, Brookhaven National Laboratory, Upton, NY) and purified as described (Grodberg & Dann, 1988) except that S-Sepharose (Pharmacia) was used instead of Trisacryl SP. Oligonucleotides were prepared at the UCSF Biomolecular Resource Center and purified as described (Ivanetich et al., 1991). [γ-^{32}P]ATP (7000 Ci/mmol) was from ICN Biomedicals, Inc. Urea–PAGE was used for RNA purification (Ogden & Adams, 1987). RUMT was purified as previously described (Gu & Santi, 1990, 1991a).

m^5U54-$tRNA^{Phe}$ was prepared by methylation of in vitro synthesized $tRNA^{Phe}$. The reaction mixture (50 μL) containing 10 μM $tRNA^{Phe}$, 1.0 μM RUMT, and 100 μM AdoMet in 50 mM Tricine, pH 8.4, 5 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 40 mM ammonium chloride, and 20 mM spermidine (Santi & Hardy, 1987) was incubated at 15 °C for 2 h; an additional 50 pmol of RUMT was added, and the incubation was continued for 2 h. The reaction mixture was extracted with an equal volume of phenol/chloroform, and

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[1] Abbreviations: FUra-$tRNA^{Phe}$, $tRNA^{Phe}$ containing substitution of Ura by FUra; m^5U54-$tRNA^{Phe}$, $tRNA^{Phe}$ containing m^5U54; AdoMet, *S*-adenosylmethionine; [^{3}H-Me]AdoMet, [*methyl*-^{3}H]AdoMet; RUMT, *Escherichia coli* tRNA (m^5U54)-methyltransferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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RNA wsa precipitated with 3 volumes of ethanol. The extent of methylation was assessed using RUMT-catalyzed methylation with [^{3}H-Me]AdoMet; the results indicated that the preparation contained less than 1% unmethylated tRNA^{Phe}.

RNA Synthesis. T7 RNA polymerase-catalyzed in vitro RNA synthesis was performed using appropriate templates and primers as described (Chu & Horowitz, 1989) (Milligan et al., 1987). Products were fractionated on 7 M urea–20% PAGE and eluted overnight with 50 mM KOAc, 20 mM EDTA, and 100 mM KCl at 4 °C. To remove urea, 1–10 A_{260} of RNA was applied to a TSK-gel Toyopearl DEAE-650 C (Supelco) column (0.5 × 0.5 cm) previously equilibrated with 20 mM Tris-HCl, pH 7.0, and 0.1 M NaCl; RNA was eluted with 0.5 M NaCl for T-arm or 0.8 M NaCl for tRNA in the same buffer and precipitated with 3 volumes of ethanol. The RNA was dissolved in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 2 mM $MgCl_2$, heated to 80 °C for 3 min, and allowed to cool to room temperature. [5-^{3}H]Ura-tRNA was prepared as above using 100 μM [5-^{3}H]UTP (3.0 Ci/mmol) and purified using Qiagen columns (Qiagen Inc.) by the procedure recommended by the manufacturer. Concentrations of tRNA were calculated using 1600 pmol per 1 A_{260} (Stanley, 1974). Concentrations of T-arm were calculated from the sum of extinction coefficients of component nucleotides with corrections for a hyperchromic effect of 1.21 (T_m = 45 °C, unpublished data); 1 A_{260} corresponded to 7400 pmol.

3′-Labeling of RNA. [5′-^{32}P]pCp was prepared using [γ-^{32}P]ATP and in vitro synthesized RNA was labeled at the 3′-end with T4 RNA ligase and [5′-^{32}P]pCp (England et al., 1980). The 3′-end-labeled RNA was purified by electrophoresis on 7 M urea–12% PAGE.

Nitrocellulose Binding Assay. Nitrocellulose filter membranes (Schleicher & Schuell, 2.4 cm) were wetted in 25 mM potassium phosphate, pH 7.4, placed on a filter manifold, and dried by gentle vacuum. Reaction mixtures contained specified amounts of RUMT and [3′-^{32}P]RNA in 50 mM Tes, pH 6.6, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM DTT, and 50 mM NaCl (binding buffer) and were incubated for the specified periods at 15 °C. Kinetic and thermodynamic constants were determined at 15 °C unless otherwise specified. Aliquots were applied to the nitrocellulose membranes, and filters were slowly washed with six 1-mL portions of 25 mM potassium phosphate, pH 7.4. Filters were counted in 5 mL of Aquasol II (Santi et al., 1974a).

SDS–PAGE Gel Shift. Gel shift assays of RUMT–RNA complexes on SDS–PAGE were performed as described (Santi & Hardy, 1987). Typically, reaction solutions containing [3′-^{32}P]RNA (ca. 500 cpm/μL) and RUMT in the binding buffer were incubated at 15 °C for specified times. A 20-μL aliquot was mixed with an equal volume of 2× loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 1.5 M 2-mercaptoethanol, 20% glycerol, and 0.005% Bromophenol blue), heated at 90 °C for 3 min, and analyzed by SDS–12% PAGE. Gels were either stained with Coomassie Blue R250 or subjected to autoradiography. For quantitation of radioactivity, bands were excised, dissolved in 1 mL of Protosol (Dupont) at 70–80 °C for 1 h, and neutralized with 0.1 mL of glacial acetic acid; the supernatants were counted in 10 mL of ACS (Amersham) (Santi & Hardy, 1987). Alternatively, gel slices were extracted with 0.5 mL of 1 M KOH at 50 °C for 5 h. After neutralization with 0.1 mL of 5 N HCl, the extracts were counted in 6 mL of Aquasol II. The amount of covalent complex formed was the same with or without heating and was completely stable through 8 min of heating at 90 °C.

Scheme I

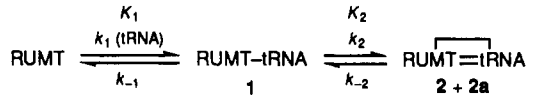


Table I: Binary Complex Formation of tRNA and T-arm with RUMT[a]

	tRNA^{Phe}	T-arm	m^{5}U54-tRNA^{Phe}	w.t. tRNA^{Phe}
k_1	6.5×10^4 M^{-1} s^{-1}	0.7×10^4 M^{-1} s^{-1}	2.0×10^4 M^{-1} s^{-1}	1.7×10^4 M^{-1} s^{-1}
k_{-1}	32×10^{-4} s^{-1} [b]	81×10^{-4} s^{-1} [b]	(200×10^{-4} s^{-1})[c]	(270×10^{-4} s^{-1})[c]
	48×10^{-4} s^{-1} [d]	76×10^{-4} s^{-1} [d]		
K_1	(8.2×10^{-8} M)[e]	(30×10^{-8} M)[e]		
	(4.9×10^{-8} M)[f]	(12×10^{-8} M)[f]		
k_2	2.5×10^{-4} s^{-1} [g]	12×10^{-4} s^{-1} [g]		
	6.6×10^{-4} s^{-1} [d]	10×10^{-4} s^{-1} [d]		
k_{-2}	4.3×10^{-4} s^{-1} [b]	10×10^{-4} s^{-1} [b]		
	6.8×10^{-4} s^{-1} [d]	14×10^{-4} s^{-1} [d]		
K_2	1.2[h]	1.7[h]		
	1.0[i]	1.4[i]		
k_{app}[j]	4.5×10^{-8} M	19×10^{-8} M	100×10^{-8} M	160×10^{-8} M

[a] Reactions were performed at pH 6.6 and 15 °C. Unparenthesized values are from a single experiment; parenthesized values are calculated from other constants. All experiments were performed two or more times, and results agreed within 10%. [b] Obtained from eq 3 with k_2 constrained to the measured value. [c] Calculated from $k_{-1} = K_1 k_1$ with K_1 from eq 1. [d] Obtained from eq 3 without constraints on k_2. [e] From eq 1. [f] From k_{-1}/k_1 using the measured k_1 and k_{-1} determined from eq 3 with k_2 constrained. [g] Measured by SDS–PAGE. [h] From SDS–PAGE measurements of [noncovalent]/[covalent] complexes at equilibrium. [i] From $K_2 = k_{-2}/k_2$ using unconstrained simulation of eq 3. [j] From nitrocellulose filter measurements of total bound complex.

RUMT Assays. tRNA methylation assays using 50 μM [^{3}H-Me]AdoMet and tritium release from [5-^{3}H]Ura-tRNA were performed at pH 6.6, 15 °C, in the binding buffer described above, or at pH 8.4, 30 °C, as described (Santi & Hardy, 1987).

RESULTS

We investigated the interactions between RUMT and unmethylated tRNA, methylated tRNA, or an unmethylated 17-base oligoribonucleotide analog of the T-arm of tRNA. Unless otherwise specified, the tRNA used was unmodified yeast tRNA^{Phe} prepared by in vitro transcription. Methylated tRNA^{Phe} was either fully modified yeast tRNA^{Phe} or m^{5}U54-tRNA prepared by RUMT-catalyzed methylation of unmodified tRNA^{Phe}. The T-arm analog was the 17mer oligoribonucleotide corresponding to nucleotides 49–65 of *E. coli* tRNA$_1^{Val}$ (5′-pppGGCGG*UUCGAUC*CCGUC) previously shown to be a substrate for RUMT (Gu & Santi, 1991b).

A minimal mechanism for the interaction of RUMT with RNA is depicted in Scheme I. First, there is reversible formation of the noncovalent RUMT–RNA binary complex **1** characterized by rate constants k_1 and k_{-1} and dissociation constant K_1. Second, there is unimolecular conversion of **1** to one or more covalent complexes (**2**, **2a**) characterized by apparent rate constants k_2 and k_{-2} and apparent equilibrium constant K_2 ([noncovalent]/[covalent]; k_{-2}/k_2). We assessed these constants both by direct measurement and by calculation from other constants (Table I).

Nitrocellulose Binding Assay. For analysis of native RUMT–RNA complexes, we adapted a nitrocellulose filter binding method commonly used to trap protein–RNA complexes (Yarus & Berg, 1970; Mougel et al., 1986; Lowary & Uhlenbeck, 1987; Su & Dubnau, 1990); as described below, this assay measures both noncovalent and covalent RUMT–RNA complexes. RUMT and [^{32}P]tRNA or the 17mer-[^{32}P]T-arm form binary complexes that can be isolated on

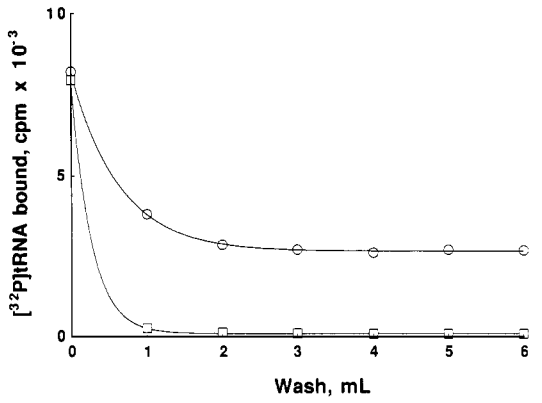


FIGURE 1: Nitrocellulose assay of RUMT–[^{32}P]tRNA complexes. The reaction mixtures (200 μL) contained 1 μM [^{32}P]tRNA^{Phe} (8 × 10^4 cpm) and binding buffer in the presence (○) or absence (□) of 0.5 μM RUMT. After incubation at 15 °C for 30 min, aliquots (20 μL) were applied to nitrocellulose filters, and the filters were washed with the indicated volumes of 25 mM potassium phosphate, pH 7.4.

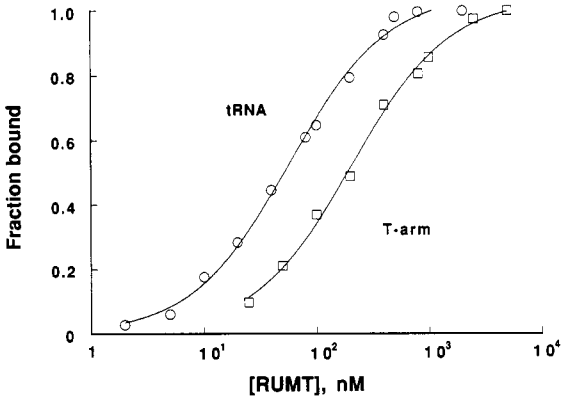


FIGURE 2: Titration of tRNA and T-arm with RUMT. Reaction mixtures (20 μL) containing 0.50 nM [^{32}P]tRNA^{Phe} (5530 cpm, ○) or 1.0 nM 17mer [^{32}P]T-arm (6020 cpm, □) and varying concentrations of RUMT were incubated at 15 °C for 60 min and assayed by nitrocellulose filtration. Data are corrected for filtration efficiencies as described in Results.

nitrocellulose filters under conditions where the free [^{32}P]-RNA is almost completely removed (Figure 1). The fact that the filter-bound RUMT–RNA complex does not decrease with continued washing indicates that the trapped bound complex does not dissociate on the filter during washing. Figure 2 shows the titration of 0.5 nM [^{32}P]tRNA^{Phe} with varying concentrations of RUMT. At saturating enzyme, 65% of the added tRNA or 60% of the T-arm was trapped. These values represent the filtration efficiency of the assays (Yarus & Berg, 1970) and are in the range described for filtration efficiencies of other protein–RNA complexes which vary between 45% and 65% (Yarus & Berg, 1970; Mougel et al., 1986; Lowary & Uhlenbeck, 1987; Su & Dubnau, 1990). Titration of RUMT with [^{32}P]tRNA results in increased bound tRNA until the enzyme is saturated. At saturation, the bound tRNA corrected for filtration efficiency corresponds to 97% of the enzyme present, indicating that 1 mol of tRNA is bound per mole of enzyme.

Apparent dissociation constants (K_{app}) for total RUMT–RNA complexes were determined by measurement of bound complexes using a constant amount of [^{32}P]RNA and varying RUMT concentration (Figure 2; Table I). The alternative use of constant RUMT and varying RNA concentration is experimentally more complicated, and titration with protein is an accepted practice in this field (Romaniuk, 1985; Lowary & Uhlenbeck, 1987; Tuerk et al., 1990). K_{app} values were

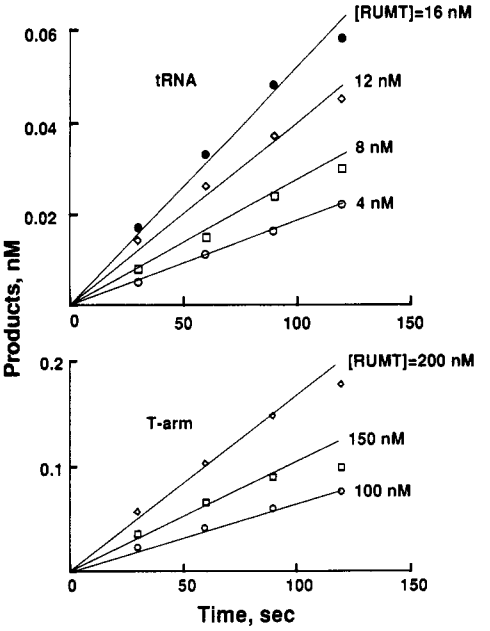


FIGURE 3: Time-dependent formation of complexes between RUMT and tRNA or T-arm. (Top) Mixtures of [^{32}P]tRNA^{Phe} (0.6 nM; 2.5 × 10^5 cpm) and varying concentrations of RUMT (4–16 nM) in binding buffer (100 μL) were kept at 15 °C; at indicated times, aliquots (20 μL) were filtered on nitrocellulose. (Bottom) Mixtures of annealed [^{32}P]T-arm (1.0 nM; 1.5 × 10^5 cpm) and varying concentrations of RUMT (100–200 nM) in binding buffer (100 μL) were kept at 15 °C; at indicated times, aliquots (20 μL) were filtered on nitrocellulose.

obtained by nonlinear least-squares fit of the binding data to an equation that calculates free ligand after correction for depletion by complex formation. The nitrocellulose assay measures both noncovalent and covalent complexes, and K_{app} is described in terms of the equilibrium and kinetic constants of Scheme I by eqs 1 and 2, respectively.

$$K_{app} = \frac{K_1 K_2}{1 + K_2} \quad (1)$$

$$K_{app} = \frac{k_{-1} k_{-2}}{k_1 (k_{-2} + k_2)} \quad (2)$$

Thermodynamic and Kinetic Constants of Native Complexes. The nitrocellulose binding assay was used to determine the bimolecular rate contant (k_1) for association of RUMT and RNA to form isolable binary complexes. Association rates were measured by mixing known amounts of excess RUMT with [^{32}P]RNA and counting the nitrocellulose-bound radioactivity. Initial rates were obtained over the first 10% of the reaction from plots of the bound RNA versus time (Figure 3), and k_1 values were calculated from the equation for a bimolecular reaction (Table I).

The dissociation of total (noncovalent + covalent) bound complexes was monitored by adding a large excess of unlabeled competitor RNA to preformed, equilibrated RUMT–[^{32}P]-RNA complexes, and measuring the bound radioactivity by the nitrocellulose filtration assay with time; in control experiments the unlabeled RNA competitor was omitted. Dissociation of the RUMT–[^{32}P]RNA complexes is in effect irreversible since, once dissociated, the [^{32}P]RNA is replaced by RNA of low specific activity. As shown in Figure 4, dissociation of the RUMT–[^{32}P]RNA complexes was biphasic. This occurs because the reaction is initiated from equilibrated covalent + noncovalent complexes, and there is an initial

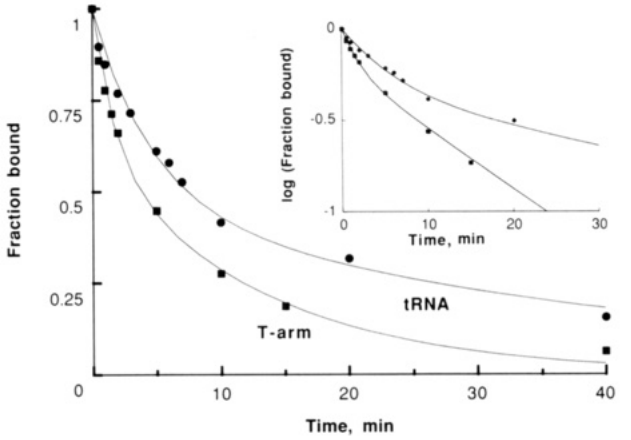


FIGURE 4: Dissociation of RUMT–tRNA and RUMT–T-arm complexes. Reaction mixtures (200 μL) contained complexes formed with 100 nM RUMT and (●) 0.5 nM [^{32}P]tRNA^{Phe} (1.2×10^5 cpm) or (■) 1 nM annealed [^{32}P]T-arm (5.8×10^5 cpm) at 15 °C. Reactions were initiated by adding 4 μL of 200 μM of cold tRNA^{Phe} or T-arm, and aliquots (20 μL) were removed at specified times for the nitrocellulose binding assay. The points are experimental and the lines are nonlinear least-squares fits of the data to eq 3. Inset: Log plot of the same data showing the biphasic nature of dissociation.

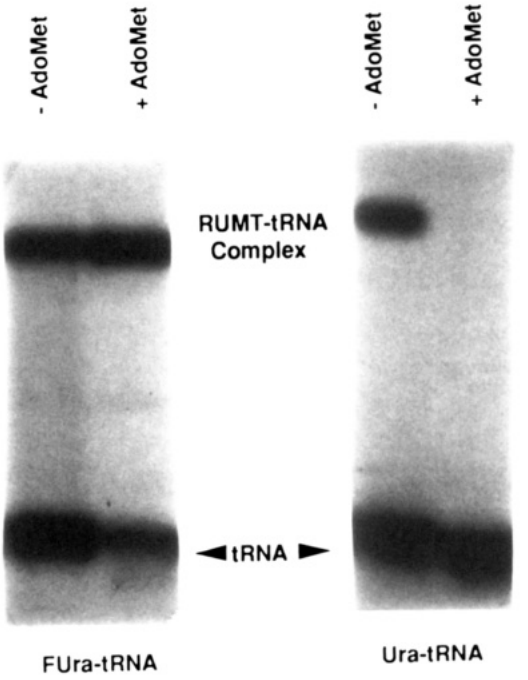


FIGURE 5: SDS–PAGE of covalent complexes between RUMT and tRNA^{Phe}, and RUMT and FUra-tRNA^{Phe} in the presence and absence of AdoMet.

relatively rapid depletion of the noncovalent complex **1** (Scheme I), followed by slower depletion of covalent complexes **2**+ **2a**. As described by Appleman et al. (1988), the irreversible dissociation of a ligand from one of two preequilibrated protein-bound forms is described by[2]

$$L_t = L_{tot}(1 - a_f e^{-k_f t} - a_s e^{-k_s t}) \quad (3)$$

where L_t is the concentration of unbound ligand at time t and L_{tot} is the concentration of total bound ligand at $t = 0$. The terms k_f and k_s are the rate constants governing the fast and slow phases, respectively, and a_f and a_s are the corresponding amplitude terms; s_k is the sum of rate constants. These terms are defined in the following equations:

$$k_f = [s_k + (s_k^2 - 4k_{-2}k_{-1})^{1/2}]/2 \quad (3a)$$

$$k_s = [s_k - (s_k^2 - 4k_{-2}k_{-1})]^{1/2}/2 \quad (3b)$$

$$s_k = k_{-2} + k_2 + k_{-1} \quad (3c)$$

$$a_f = \frac{k_s(k_{-2} + k_2 - k_f)}{(k_{-2} + k_2)(k_s - k_f)} \quad (3d)$$

$$a_s = \frac{k_f(k_{-2} + k_2 - k_s)}{(k_{-2} + k_2)(k_f - k_s)} \quad (3e)$$

The dissociation of RUMT–RNA complexes was simulated by a nonlinear least-squares fit of the data to eq 3 (Figure 4). In one simulation, we used the experimentally determined values for k_2, and fitted k_{-2} and k_{-1}; we also simulated the reaction without applying constraints on k_2. The simulated k_2 value for the RUMT–T-arm complex was in excellent agreement with the value obtained from SDS–PAGE measurements (above), whereas the simulated k_2 for the RUMT–tRNA complex was about 40% of that from the SDS–PAGE measurements. The values obtained for k_{-2} and k_{-1} were in acceptable agreement with values calculated using constants measured by different methods. From K_1 obtained from eq 1 and the measured k_1, and $k_{-1} = K_1 k_1$, we calculated k_{-1} values of 5.3×10^{-3} s^{-1} for tRNA and 2.1×10^{-3} s^{-1} for the T-arm; from measured values of k_2 and K_2, and $k_{-2} = k_2 K_2$, we calculated k_{-2} values of 3.0×10^{-4} s^{-1} for tRNA and 2.0×10^{-3} s^{-1} for the T-arm.

Covalent RUMT–RNA Complexes. To monitor covalent RUMT–RNA complexes, we adapted the SDS–PAGE gel shift assay (Santi & Hardy, 1987). The basis of this assay is that when RNA is covalently bound to the 42-kDa RUMT, migration of the complex is retarded on SDS–PAGE.

As previously described, RUMT, FUra-tRNA, and [^{3}H-Me]AdoMet react to form a covalent complex which migrates on SDS–PAGE as a 61-kDa protein, contains tritium, and stains with Coomassie blue (Santi & Hardy, 1987). In the present work, we have found that, in the absence of AdoMet, [^{32}P]FUra-tRNA and RUMT also form a covalent adduct which is stable to denaturation and SDS–PAGE (Figure 5). The complex migrates as a 63-kDa protein which contains ^{32}P and stains with Coomassie Blue. RUMT and unmodified tRNA also form complexes stable to denaturation (2% SDS at 90 °C for at least 8 min) and SDS–PAGE. Autoradiography revealed the free [^{32}P]tRNA migrating at about 20 kDa with respect to protein standards, and a band migrating at about 63 kDa (Figure 5). Coomassie Blue staining reveals both free protein at 42 kDa and protein comigrating with radioactivity derived from [^{32}P]tRNA at 63 kDa. In similar experiments, we showed that RUMT and the [^{32}P]T-arm formed a complex which migrated as a 47-kDa protein. After these RUMT–RNA complexes were treated with RNase A (50 μg/mL, 1 h at 37 °C), the bands moved as 42-kDa proteins and did not contain ^{32}P. In the presence of AdoMet, no covalent adducts are observed, since complexes are converted to products and free enzyme. We have estimated that, under the conditions used, the rate of formation of the RUMT–tRNA covalent complex is about one-half that of the RUMT–FUra-tRNA covalent complex.

The binary RUMT–tRNA complexes are composed of both noncovalent and covalent bound components. Figure 6 shows

[2] In addition to its importance for tight binding enzyme inhibitors, eq 3 seems particularly relevant in analysis of the dissociation of protein–nucleic acid complexes which show components of loose nonspecific binding followed by tight specific binding. It is noted that a single experiment to measure progress of dissociation provides estimates of all constants of the interaction except k_1 and, hence, K_1.

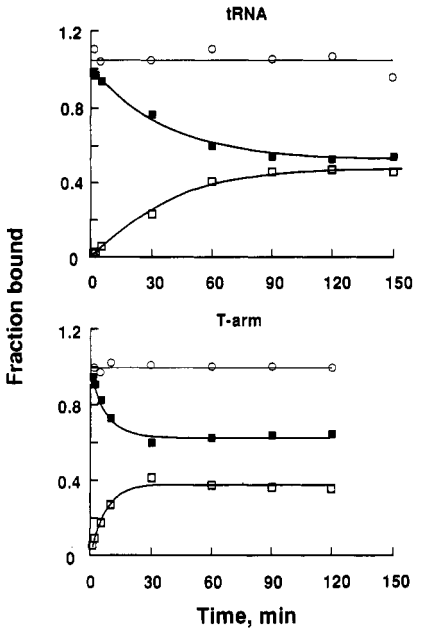


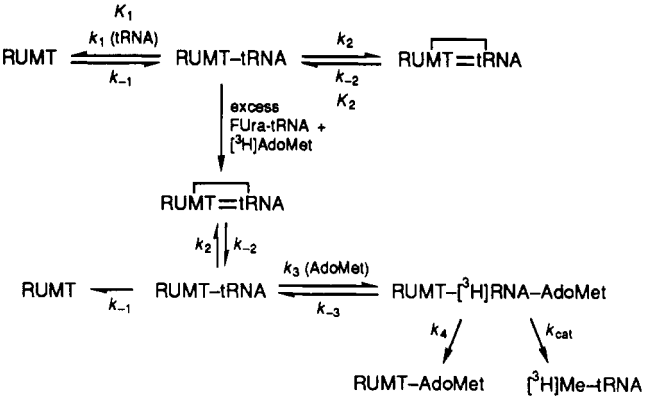
FIGURE 6: Covalent complex formation between RUMT and in vitro synthesized tRNA or T-arm versus time. The reaction mixtures (200 μL) contained 50 nM [^{32}P]tRNA^{Phe} (1.9 × 10^5 cpm) and 1 μM RUMT, or 50 nM [^{32}P]T-arm (2.4 × 10^5 cpm) and 8 μM RUMT at 15 °C. Aliquots (20 μL) were removed at the indicated times and assayed by nitrocellulose filtration (total complex) (○) or SDS–PAGE (covalent complex) (□). Noncovalent complex (■) was calculated as the difference between the total and covalent complexes. Experiments performed using 5 nM [^{32}P]tRNA^{Phe} or [^{32}P]T-arm (not shown) gave similar results.

an experiment in which [^{32}P]tRNA was treated with excess RUMT (20K_d), and aliguots were removed at varying times for analysis by both nitrocellulose filter assay and SDS–PAGE. The total enzyme-bound tRNA (covalent + noncovalent) is measured by the nitrocellulose assay, and the covalent component by the SDS–PAGE assay. The noncovalent complex is estimated as the difference between total and covalent complexes or by quantitating the free tRNA on SDS–PAGE at saturating enzyme. As shown, the complex is initially composed only of noncovalent complex and RNA is completely bound to RUMT throughout the experiment. With time, the covalent complex increases to about 45% of the total bound tRNA, and the noncovalent complex decreases to about 55% of the total whereafter there are no further changes; thus, the ratio of noncovalently to covalently bound [^{32}P]tRNA, K_2, was 1.2. In a similar experiment with RUMT and the T-arm, the ratio of noncovalent to covalent [^{32}P]T-arm was 1.7.

The initial rates of formation of the covalent complexes were assessed by mixing tRNA or T-arm (5 and 50 nM) with excess amounts of RUMT at 15 °C, and quantitating the covalent complex after SDS–PAGE at varying times (Figure 6). At the concentrations of components used, the formation of noncovalent complexes was rapid, and k_2 could be measured as the subsequent appearance of covalent complex (Table I). The first-order rate constants (k_2) calculated for disappearance of noncovalent complexes in the same experiments were in good agreement (2.2 × 10^{-4} s^{-1} for tRNA and 1.0 × 10^{-3} s^{-1} for T-arm).

Interaction of RUMT with Methylated tRNA. We also studied the interaction of RUMT with tRNA which was methylated at U54, using m^{5}U54-tRNA prepared by RUMT methylation of in vitro transcribed tRNA^{Phe}, or wild-type tRNA^{Phe} which contains a complete complement of modified bases. The rate constants (k_1) for association of the methylated tRNAs with RUMT were 3- to 4-fold lower than the rate

Scheme II



constant for unmodified tRNA. The rate constants for dissociation were too rapid to measure by the method used, but could be calculated (Table I); they are about 6- to 9-fold higher than those obtained with unmethylated RNA counterparts. The methylated RNAs bound to RUMT with K_{app} values 22- to 35-fold higher than unmodified tRNA. Covalent complexes were not detected by SDS–PAGE, but this may be due to rapid rates of dissociation.

Isotope Trapping of the Binary Complexes. As depicted in Scheme II, an isotope trapping or "pulse–chase" experiment (Rose et al., 1974) was performed to ascertain whether the covalent complexes could be converted to methylated product without dissociation and reassociation. In effect, these experiments measure partitioning of the binary RUMT–RNA complexes between dissociation and product formation during a single turnover. The principle of the experiment is as follows. An equilibrated "pulse" solution of RUMT and [^{32}P]tRNA (or T-arm) was added to a "chase" solution containing excess [^{3}H-Me]AdoMet and the RUMT inhibitor FUra-tRNA. In the chase, unbound enzyme is inactivated by the excess FUra-tRNA. If the binary complexes dissociate (k_{-1}), the resultant free RUMT reacts with excess FUra-tRNA and is inactivated. If the binary complexes form a productive ternary complex with [^{3}H-Me]AdoMet (k_3), the RUMT–RNA–AdoMet complex may (a) undergo dissociation of AdoMet (k_{-3}) and revert to the binary RUMT–[^{32}P]RNA complex, (b) form tritiated methyl product (k_{cat}), or (c) undergo dissociation of RNA (k_4) to form the RUMT–AdoMet complex which would react with FUra-tRNA faster than with [^{32}P]tRNA. Thus, providing there is sufficient quenching of free enzyme and substrate, there is only one route by which the covalent RUMT–RNA complexes can form [^{3}H-Me]Me-RNA (i.e., through k_{-2} and k_{cat}), and the amount of [^{3}H-Me]Me-RNA formed measures passage through that route. The ^{3}H/^{32}P ratio in the total free RNA provides a convenient measurement of the fraction of the bound [^{32}P]tRNA which is converted to product [i.e., [product]/([substrate] + [product])]; high ratios indicate partitioning to Me-tRNA while low ratios indicate dissociation of the binary complex before methylation.

In a control experiment to show that the FUra-tRNA in the chase solution was sufficient to prevent free enzyme from catalyzing methylation, RUMT was added to a solution which contained [^{32}P]tRNA, FUra-tRNA, and [^{3}H-Me]AdoMet, all in concentrations simulating the isotope trapping experiment described below; after quenching at 60 min, there was no [^{3}H-Me]Me-tRNA in the tRNA fraction and no [^{32}P]-tRNA in the covalent complex. This demonstrates that, under these conditions, free enzyme is trapped as the covalent RUMT–FUra-tRNA complex faster than it catalyzes methylation of tRNA.

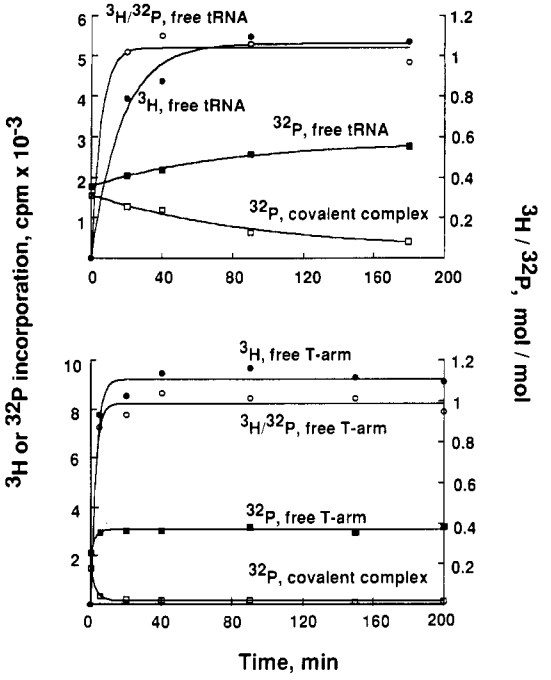


FIGURE 7: Isotope trapping of RUMT–RNA binary complexes. Complexes were formed from 100 μL of 0.4 μM [^{32}P]tRNA^{Phe} (1.3 × 10^5 cpm) and 2 μM RUMT, or 120 μL of 0.4 μM [^{32}P]T-arm (1.0 × 10^5 cpm) and 10 μM RUMT in binding buffer at 15 °C; aliquots (10 μL) were assayed by nitrocellulose filtration and by SDS–PAGE. For isotope trapping, 50 μL of the tRNA^{Phe} mixture or 80 μL of the T-arm mixture was mixed with one-half volume of a solution containing 48 μM FUra-tRNA^{Phe} and 20 μM [^{3}H-Me]AdoMet (10 Ci/mmol) (final concentrations: 0.27 μM [^{32}P]tRNA or 0.27 μM [^{32}P]T-arm, 1.3 μM RUMT or 6.7 μM RUMT, 16 μM FUra-tRNA, 6.7 μM [^{3}H-Me]AdoMet) and kept at 15 °C. Aliquots (15 μL) were withdrawn at specified times and subjected to SDS–PAGE. Bands containing ^{32}P were located by autoradiography, excised, extracted, and counted for ^{32}P and ^{3}H; (●) ^{3}H in free RNA; (○) ^{3}H/^{32}P in free RNA; (■) ^{32}P in free RNA; and (□) ^{32}P in the covalent complexes. Free RNA is a composite of both SDS–PAGE-unstable noncovalently bound and unbound species. For controls, solutions were made containing all components as above except RUMT; RUMT was added, and after 60 min at 15 °C reactions were analyzed by SDS–PAGE.

A binary complex was formed using excess RUMT (2 μM) and [^{32}P]tRNA (0.4 μM); nitrocellulose assay showed that >95% of the [^{32}P]tRNA was bound, and SDS–PAGE analysis showed that 46% of the bound complex was covalently bound. This solution was added to the "chase" solution containing [^{3}H-Me]AdoMet and exess FUra-tRNA (60-fold over [^{32}P]-tRNA, 12-fold over enzyme). Aliquots were removed with time (over 7 half-lives of k_{-2} for tRNA, 20 half-lives of k_{-2} for T-arm) and analyzed by SDS–PAGE; ^{3}H and ^{32}P in bands corresponding to the RUMT complex and RNA were quantitated. As shown in Figure 7 the [^{3}H-Me]Me-tRNA product formed was equal to the tRNA which was bound to RUMT as the binary complex, and the ^{3}H/^{32}P ratio indicated that all substrate was converted to product. Even though the covalent complex comprised only 46% of the initial binary complex, within experimental error, it was completely converted to product. A similar experiment was performed with the [^{32}P]T-arm, with similar results Figure 7; i.e., the covalent complex is methylated without undergoing dissociation.

Steady-State Kinetics. The kinetic parameters for RUMT-catalyzed methylation of tRNA^{Phe} and the T-arm under the pH 6.6 conditions used for the binding assays are given in Table II. Using the pH 8.4 conditions of the previously described assay (Santi & Hardy, 1987) tRNA^{Phe} showed a K_m of 0.8 μM and k_{cat} of 0.09 s^{-1}, and the T-arm showed a K_m of 5 μM and k_{cat} of 0.03 s^{-1}.

Table II: Steady-State Kinetic Parameters of RUMT[a]

	methylation with AdoMet			5-[^{3}H] exchange
substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})
tRNA	0.31×10^{-6}	0.030	9.7×10^4	4.2×10^{-4}
T-arm	2.0×10^{-6}	0.011	0.55×10^4	

[a] Reactions were performed at pH 6.6 and 15 °C.

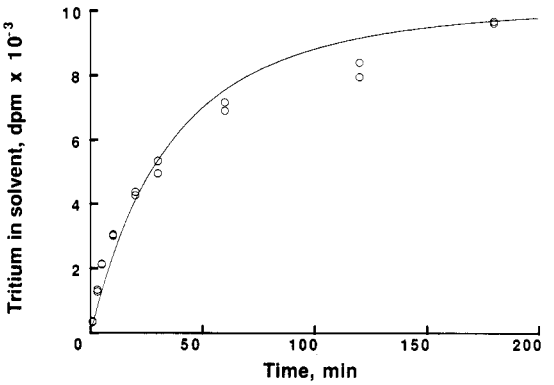
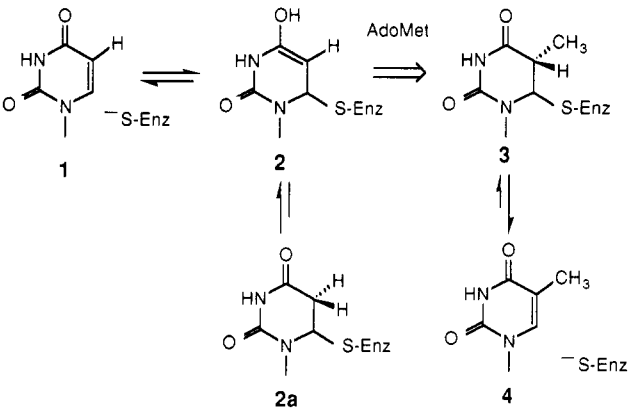


FIGURE 8: Tritium release from [5-^{3}H]Ura-tRNA^{Phe} into water catalyzed by RUMT. The reaction (1500 μL) contained 1 μM [5-^{3}H]-Ura-tRNA^{Phe} (6400 dpm/pmol) and 100 nM RUMT in binding buffer. Aliquots (50 μL) were removed as specified for assay of tritium release into solvent (Santi & Hardy, 1987).

Scheme III



RUMT catalyzes the exchange of tritium from the 5-position of U54 of total m^{5}U-deficient tRNA for protons of water, showing k_{cat} values of 9×10^{-4} s^{-1} at 30 °C and pH 8.4 (Santi & Hardy, 1987). We prepared [5-^{3}H]Ura-tRNA^{Phe} by in vitro synthesis using [5-^{3}H]UTP. In the absence of AdoMet, RUMT catalyzed the release of 0.7 equiv of tritium into water with a k_{cat} of 4.2×10^{-4} s^{-1} at 15 °C and pH 6.6 (Figure 8).

DISCUSSION

The proposed mechanism for the RUMT-catalyzed methylation of RNA is shown in scheme III. After formation of reversible complexes, Cys 324 or RUMT adds to the 6-carbon of the target U54 of tRNA to activate the 5-position of the heterocycle toward electrophilic substitution. The enol (or equivalent enolate) intermediate **2** accepts the methyl group of AdoMet in an irreversible step to give **3**, which upon β-elimination provides the methylated RNA and unmodified enzyme. In the absence of AdoMet, the 5-hydrogen of the substrate undergoes a slow exchange with protons of solvent. The 5-H exchange reaction requires formation of a 5,6-dihydropyrimidine intermediate, **2a**, in which two hydrogens are bound to the 5-carbon (Pogolotti et al., 1979; Santi & Hardy, 1987). This intermediate is not on the direct reaction

pathway to methylation but may be formed by tautomerization of intermediate **2**.

In the present work, we have shown that RUMT forms binary covalent complexes with unmethylated tRNA and a 17mer analog of the T-arm of tRNA in the absence of AdoMet. Both noncovalent and covalent complexes can be trapped on nitrocellulose filters, and the covalent complexes can be isolated on SDS–PAGE. A covalent binary complex between RUMT and FUra-tRNA can also be isolated on SDS–PAGE.

The competence of the binary covalent complexes to convert to products was shown by an isotope trapping procedure (Rose et al., 1974). When preformed RUMT–tRNA (or T-arm) complexes were presented with AdoMet under conditions which permit only a single turnover, all RNA was converted to methylated product without dissociation of the complex. Hence, both noncovalent and covalent complexes are *chemically* competent precursors to the methylated RNA product. This experiment further shows that RNA does not directly dissociated from the ternary complex via k_4 (Scheme II).

The structure of the covalent RUMT–RNA complexes is assigned from precedence, experimental evidence, and chemical considerations. The precedence is provided by the enzyme thymidylate synthase which forms covalent complexes analogous to **2** and **2a** with dUMP and 5-fluoro-2′-deoxyuridylate (Santi et al., 1974b; Moore et al., 1986). The experiments described here show that the binary covalent complex is both reversible and capable of being methylated. Although both **2** and **2a** fulfill these requirements, **2** is expected to be highly reactive, and to tautomerize to **2a** upon denaturation of the protein. Further, the existence of **2a** is required to explain the exchange of the 5-H of U54 with solvent protons demonstrated here and elsewhere (Santi & Hardy, 1987). Finally, as discussed below, the isolable covalent complex **2a** is a kinetically competent intermediate in the 5-H exchange reaction, whereas it is chemically competent but kinetically incompetent in the methylation reaction; **2a**, but not **2**, fulfills these criteria. Considering all aspects, the Michael adduct **2a** is the most likely structure of the isolable covalent binary complex.

Using the nitrocellulose and SDS–PAGE binding assays, we have determined the complete kinetics and thermodynamics of the interaction of RUMT with RNA substrates and products (Table I). Formation of binary covalent RUMT–RNA adducts **2a** does not provide a large thermodyanmic advantage for binary complex formation; in fact, noncovalent complexes **1** predominate at equilibrium (K_2 = 1.2–1.7). However, the covalent complex is slowly formed, and once formed it slowly reverses ($t_{1/2} \sim$ 10 min for T-arm, to 20 min for tRNA). Thus many of unusual properties of the covalent complexes reflect their kinetic rather than thermodynamic properties. Below, we interpret the data on the binding and kinetics of the RUMT–RNA complexes in the context of the steady-state kinetic parameters of the RUMT-catalyzed methylation and 5-H exchange reactions.

The k_{cat} of an enzymic reaction reflects the slowest unimolecular rate constant in the pathway [Fersht (1985), p 150]. In the proposed mechanism in Scheme III, the exchange of the 5-H of U54 for protons of water in the absence of AdoMet demands formation and reversal of intermediate **2a** at a rate equal to or greater than that of 5-H exchange (Pogolotti et al., 1979). We have shown that the 5-H exchange proceeds at a rate which is comparable to or slower than any unimolecular reactions (k_2, k_{-2}, and k_{-1}) involved in the formation and reversal of the covalent complex (Table I). Hence, the formation of **2a** is kinetically competent as an intermediate in the 5-H exchange reaction catalyzed by RUMT in the absence of AdoMet. However, the formation (k_2) and disruption (k_{-2}) of the RUMT–tRNA covalent complex **2a** are about 100-fold slower (about 10-fold with the T-arm) than k_{cat} for the methylation reaction; thus, the isolated covalent intermediate **2a** is kinetically *incompetent* as an intermediate in the normal methylation reaction when AdoMet is available. We conclude that **2a** is not on the normal pathway to products, and it is not an essential intermediate in the methylation reaction.

Dissociation constants (K_1) for noncovalent binary complexes containing tRNA or T-arm are significantly lower than the corresponding apparent K_m values in the methylation reaction. Since k_{cat} for the methylation reaction of tRNA is significantly faster than the rate of dissociation of the noncovalent complex (k_{-1}), it is concluded that once tRNA is bound in the RUMT–tRNA–AdoMet Michaelis complex, it is committed to reaction.

The specificity constant k_{cat}/K_m measures the slowest bimolecular rate constant in an enzymic reaction [Fersht (1985), p 104]. Interestingly, the bimolecular rate constants (k_1) for RUMT and tRNA or the T-arm are similar to the corresponding k_{cat}/K_m values for methylation. This suggests that the specificity constants reflect binding of RNA to RUMT. However, k_1 is quite low compared to rate constants for other protein–RNA interactions reported which range over 10^5–10^8 M^{-1} s^{-1} (Krauss et al., 1973; Pingoud et al., 1973; Romaniuk, 1985; Mougel et al., 1986; Lowary & Uhlenbeck, 1987; Su & Dubnau, 1990). Indeed, interactions of tRNA with aminoacyl tRNA synthetases occur near diffusion control limits [Fersht (1985), p 106]. Possibly, there are weaker complexes formed prior to the one we measure, or that only a subset of the free RNA molecules are in a conformation suitable for binding to RUMT; either model would explain the low rate constants for association. A two-state binding or conformer-equilibrium model for RNA would also explain the higher k_1 value observed with tRNA compared to the T-arm; here, the initial complex formed with the T-arm would be weaker, or the pool of free T-arm with an appropriate conformation for binding would be smaller.

It is interesting that covalent complex formation between RUMT and the methylated tRNA product (intermediate **3** in Scheme III) is undetectable by the methods used here; however, it exists in the mechanism proposed in Scheme III, and we believe it is simply too unstable to isolate. Further, the binding of RUMT to methylated tRNA products (K_{app}) is 22- to 35-fold weaker than to unmethylated substrates, and the rate of dissociation of the binary complex is about 6- to 9-fold faster for methylated tRNA. These data suggest that once the methylated RNA product is formed, it is favorable to disrupt the covalent adduct and release the product from the enzyme, thus leaving the enzyme available for catalysis.

Relevance of RUMT–RNA Complexes in Vivo. Recent data indicate that, in *E. coli* cells expressing RUMT, much of the enzume is covalently complexed to RNA (Ny et al., 1988; Gu & Santi, 1990), indicating that covalent binary complexes may be relevant in vivo. From the results reported here, the complex could serve as a depot of the enzyme–tRNA complex awaiting interaction with AdoMet. However, it is not known whether tRNA is the only species bound to RUMT in vivo, and it is difficult to rationalize why a depot of RUMT–tRNA would be advantageous. Recently, we have suggested that RUMT may be capable of recognizing RNAs other than tRNA (Gu & Santi, 1991b), and it has been found that 16S RNA is also covalently bound to RUMT in *E. coli* (Bjork,

unpublished data) and methylated by RUMT (Gu and Santi, unpublished data). Thus, covalent Michael adducts may form between RUMT and RNAs other than tRNA and may play a role which is thus far unknown.

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