Catalytic Mechanism and Inhibition of tRNA (Uracil-5-)methyltransferase: Evidence for Covalent Catalysis†

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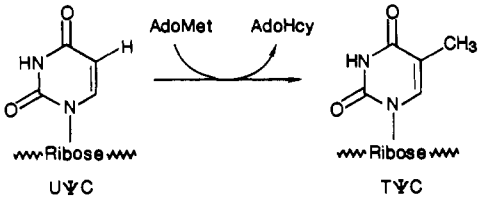
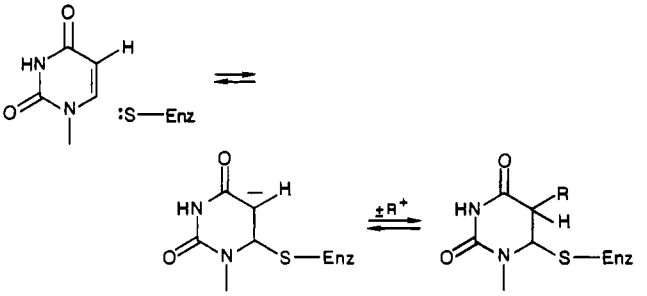
ABSTRACT: tRNA (Ura-5-)methyltransferase catalyzes the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to the 5-carbon of a specific Urd residue in tRNA. This results in stoichiometric release of tritium from [5-^{3}H]Urd-labeled substrate tRNA isolated from methyltransferase-deficient *Escherichia coli*. The enzyme also catalyzes an AdoMet-independent exchange reaction between [5-^{3}H]-Urd-labeled substrate tRNA and protons of water at a rate that is about 1% that of the normal methylation reaction, but with identical stoichiometry. *S*-Adenosylhomocysteine inhibits the rate of the exchange reaction by 2–3-fold, whereas an analogue having the sulfur of AdoMet replaced by nitrogen accelerates the exchange reaction 9-fold. In the presence (but not absence) of AdoMet, 5-fluorouracil-substituted tRNA (FUra-tRNA) leads to the first-order inactivation of the enzyme. This is accompanied by the formation of a stable covalent complex containing the enzyme, FUra-tRNA, and the methyl group of AdoMet. A mechanism for catalysis is proposed that explains both the 5-H exchange reaction and the inhibition by FUra-tRNA: the enzyme forms a covalent Michael adduct with substrate or inhibitor tRNA by attack of a nucleophilic group of the enzyme at carbon 6 of the pyrimidine residue to be modified. As a result, an anion equivalent is generated at carbon 5 that is sufficiently reactive to be methylated by AdoMet. Preliminary experiments and precedents suggest that the nucleophilic catalyst of the enzyme is a thiol group of cysteine. The potent irreversible inhibition by FUra-tRNA suggests that a mechanism for the "RNA" effects of FUra may also involve irreversible inhibition of RNA-modifying enzymes.

The enzyme tRNA (Ura-5-)methyltransferase (EC 2.1.1.35) catalyzes the *S*-adenosylmethionine (AdoMet)[1] dependent methylation of a specific Urd residue to form the m^{5}U (ribosylthymine) residue found in the TΨC loop (loop IV) of most eubacterial and eukaryotic tRNAs thus far sequenced (Sprinzl et al., 1985). The similarity of this enzyme to others



that catalyze one-carbon transfer to carbon 5 of pyrimidine substrates [such as thymidylate synthase (TS), dUMP hydroxymethyltransferase, and DNA (Cyt-5-)methyltransferase] suggests they may share mechanistic features. The first step in the reaction catalyzed by TS, the paradigm for this class of enzymes, is production of a 5,6-dihydropyrimidine intermediate. Michael addition of a cysteine thiol of the enzyme to carbon 6 of dUMP generates a covalent enzyme–substrate intermediate and serves to activate the 5-position for reaction with the electrophilic one-carbon unit of the cofactor, 5,10-methylenetetrahydrofolate. The covalent adduct can also react with water which results in direct exchange of the 5-H of dUMP for protons of solvent. Similar covalent bonds are formed with the inhibitor FdUMP, resulting in a stable covalent adduct composed of TS, FdUMP, and the cofactor (Santi & Danenberg, 1984).



It has been observed that tRNA in which Ura is extensively replaced by FUra is an inhibitor of tRNA (Ura-5-)methyltransferase activity, both in vivo (Frendewey & Kaiser, 1979; Randerath et al., 1983) and in vitro (Frendewey et al., 1982). By analogy with TS, these results suggested to us that tRNA (Ura-5-)methyltransferase catalysis and inhibition by FUra-tRNA may proceed via nucleophilic catalysis. In the present work, we show that, in the *absence* of AdoMet, tRNA (Ura-5-)methyltransferase catalyzes proton exchange between

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[1] Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMDB, N^4-adenosyl-N^4-methyl-2,4-diaminobutanoic acid; AdoMet, *S*-adenosylmethionine; EDTA, ethylenediaminetetraacetic acid; FdUMP, 5-fluoro-2′-deoxyuridylate; FUra, 5-fluorouracil; FUra-tRNA, tRNA containing extensive substitution of Ura residues by FUra; FUrd, 5-fluorouridine; HPLC, high-performance liquid chromatography; m^{5}C, 5-methylcytidine; m^{5}U, 5-methyluridine (ribosylthymine); PAGE, polyacrylamide gel electrophoresis; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); RNase, ribonuclease; SDS, sodium dodecyl sulfate; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; TS, thymidylate synthase.

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[5-^{3}H]Urd-tRNA and solvent water. We also show that, in the presence of AdoMet, FUra-tRNA causes irreversible inactivation of the enzyme which is accompanied by the formation of a stable covalent complex between the enzyme, FUra-tRNA, and the methyl group of AdoMet. It appears that FUra-tRNA behaves as a mechanism-based inhibitor of the enzyme, which may be relevant in the action of the drug FUra. From these results, we can deduce certain features of the mechanisms of catalysis and inhibition of tRNA (Ura-5-)methyltransferase which may apply to certain other enzymes that modify RNA.

Materials and Methods

Tricine and spermidine tris(hydrochloride) were products of U.S. Biochemical Corp. Tes, Pipes, *S*-adenosyl-L-homocysteine (AdoHcy), and L-AdoMet·HCl were obtained from Sigma Chemical Co. DEAE-cellulose was DE-52 from Whatman, and *p*-(chloromercuri)benzoate was from ICN Biochemicals. N^4-Adenosyl-N^4-methyl-2,4-diaminobutanoic acid (AdoMDB), an analogue of AdoMet having the sulfur replaced by nitrogen, was provided to us by Albert Minnick and George Kenyon. [5-^{3}H]Urd (26 Ci/mmol) was purchased from Moravek Biochemicals. [^{14}C]Methyl-AdoMet (60 Ci/mol) and [^{3}H]methyl-AdoMet (0.5-15 Ci/mmol) were purchased from Amersham. *Escherichia coli* GB1-5-39, a strain deficient in tRNA (Ura-5-)methyltransferase, and the same strain containing the plasmid pTN102, which carries the gene for tRNA (Ura-5-)methyltransferase (Ny & Bjork, 1980), were generously provided to us by Glenn Bjork of the University of Umea, Umea, Sweden. Wild-type tRNA from *E. coli* B was obtained from Schwarz/Mann. Protein concentrations were measured by the dye-binding method of Bradford (1976) calibrated by using bovine serum albumin.

Preparation and Analysis of tRNAs. E. coli GB1-5-39, a methylase-deficient strain, was used to prepare tRNA lacking m^{5}U. Bulk unlabeled m^{5}U-deficient tRNA was purified (Buck et al., 1983) from cells grown in the rich nutrient medium described by Ny and Bjork (Ny & Bjork, 1980) [LB (Bertani, 1951) made up in medium E of Vogel and Bronner (1956) plus 0.5% glucose]. Concentrations of tRNA were calculated on the basis of a 1 mg/mL solution of tRNA having an A_{260} of 24, and by use of average molecular weight for tRNA of 26 000 (Stanley, 1974). The nucleoside composition of tRNA was determined by HPLC analyses of enzymatically digested samples as described (Buck et al., 1983).

m^{5}U-deficient tRNA labeled with [5-^{3}H]Urd was prepared by growing *E. coli* GB1-5-39 in the minimal salts medium of Vogel and Bronner (1956) supplemented with 0.5% glucose, 20 μg/mL arginine, and 1 μg/mL thiamin and containing 20 μCi/mL [5-^{3}H]Urd (26 Ci/mmol). This medium (250 mL) was inoculated with 2 mL of an overnight culture and grown with shaking at 37 °C. Labeled cells were harvested by centrifugation after 9.5 h of growth, when A_{600} for a parallel unlabeled culture was 1.5. The bulk labeled tRNA, purified by the method of Buck et al. (1983), had a specific activity of 14 μCi/A_{260} unit (8.7 Ci/mmol) for one preparation and 5.1 μCi/A_{260} unit (3.2 Ci/mmol) for another.

tRNA in which most of the Urd residues were replaced by FUrd was prepared from *E. coli* GB1-5-39 in the minimal salts medium containing FUra (Horowitz & Chargaff, 1959) and dThd. For a typical preparation, 1 L of the minimal medium was inoculated with 50 mL of an overnight culture and growth with shaking at 37 °C. When the A_{600} of the culture reached 0.3, additions were made of 2-mL solutions of FUra in 0.25 M potassium carbonate and thymidine in distilled water sufficient to provide 10 μg of FUra and 25 μg of thymidine per milliliter of growth medium. After 3-h incubation at 37 °C, cells were harvested and bulk FUra-tRNA was prepared by the method of Buck et al. (1983). The tRNA had 70% of its Urd residues replaced by FUrd, as measured by HPLC analysis of a sample digested to nucleosides (Buck et al., 1983).

The stoichiometry of in vitro methylation of the unlabeled tRNA isolated from *E. coli* GB1-5-39 was determined as follows. A sample of 1.5 nmol of tRNA was incubated for 3 h at 37 °C in 0.29 mL of the assay buffer described below containing 800 units of enzyme and 500 μCi/mL (17 μM) [^{3}H]methyl-AdoMet (14.5 Ci/mmol). The reaction mixture was diluted to 1 mL with distilled water and applied to a 0.5-mL column of DEAE-cellulose previously equilibrated with TM buffer (10 mM Tris·HCl, 10 mM $MgCl_2$ pH 7.5). The column was washed with 11.5 mL of TM buffer and then 15 mL of 0.22 M KCl in TM buffer, to remove protein and excess radiolabeled AdoMet. The labeled tRNA was eluted from the column with 1.2 mL of 1 M KCl in TM buffer, and its specific activity was determined by measurement of the A_{260} value of the tRNA solution and scintillation counting.

tRNA (Ura-5-)methyltransferase Assays. The activity of the methyltransferase was assayed in two ways. For routine assays during purification of the enzyme the method used monitored the incorporation of tritium from [^{3}H]methyl-AdoMet into m^{5}U-deficient tRNA (Greenberg & Dudock, 1980), with slight modifications. The assay buffer contained 50 mM tricine, pH 8.4, 5.0 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 40 mM ammonium chloride, and 20 mM spermidine. The concentrations of m^{5}U-deficient tRNA and [^{3}H]methyl-AdoMet (15 Ci/mmol) used in the assay mixture were 7.7 and 1.7 μM, respectively. Five microliters of a 10 mg/mL solution of wild-type *E. coli* B tRNA was added to each 20-μL assay mixture just prior to quenching with 1 mL of cold 10% trichloroacetic acid. The precipitated tRNA was filtered onto a glass fiber disk (Whatman GF/C), rinsed 5 times with 2 mL of cold 2% trichloroacetic acid, and dried by washing with several milliliters of 100% ethanol. Disks were counted in 10 mL of 0.4% Omnifluor (New England Nuclear) in xylene. One unit is the amount of enzyme that catalyzes the methylation of 1 pmol of tRNA/min at 30°, under the standard reaction conditions.

The second assay of the methyltransferase monitored upon tritium release from [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA accompanying methylation. In a typical reaction, a solution containing 125 μM AdoMet, 0.6 μM labeled tRNA, and enzyme, in assay buffer, was incubated at 30 °C. Aliquots of 100 μL were quenched at timed intervals by addition to 1 mL of 5% Norit A in 0.1 N HCl. Each quenched sample was centrifuged briefly in an Eppendorf microcentrifuge, and 0.9 mL of the supernatant was removed and again mixed with 0.5 mL of 5% Norit A in 0.1 N HCl. This mixture was centrifuged for several minutes, the resulting supernatant was filtered through a glass wool plug in a Pasteur pipet, and 1.1 mL of the filtrate was counted in 10 mL of Aquasol-2 (New England Nuclear). The volumes of the reaction aliquot and quench solution employed depended on the sensitivity required in the assay. For example, reactions containing 0.1 μM tRNA were monitored by quenching 1-mL samples with 0.3 mL of 10% Norit A in 0.4 N HCl, centrifugation, and proceeding with the second charcoal treatment as described above. Two treatments with charcoal were routinely necessary to remove nucleotide-bound tritium. Tritium release catalyzed by the methyltransferase in the absence of AdoMet and in the presence of analogues of AdoMet was monitored by an identical procedure.

The rates of methylation and tritium release were compared directly by double-label experiments using [^{14}C]methyl-AdoMet to monitor methylation and [5-^{3}H]Urd-labeled substrate tRNA to monitor tritium release. A reaction mixture was prepared in 1.5 mL of assay buffer containing 65 μM [^{14}C]methyl-AdoMet (60 mCi/mmol), 15 μM unlabeled m^{5}U-deficient tRNA, 2.0 μM [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA (3.2 Ci/mmol), and 17 units of enzyme. Appearance of tritium in solvent was monitored by the charcoal absorbtion assay described above. Incorporation of [^{14}C]-methyl into tRNA was monitored by the following procedure. At timed intervals, 20-μL aliquots were mixed with 0.2 mL of a 50% aqueous slurry of DEAE-cellulose (chloride form) containing 20 mM mercury acetate which totally inhibits the methyltransferase activity. The DEAE-cellulose containing the adsorbed tRNA was pelleted by centrifugation, and the supernatant was removed by aspiration. The resin was washed 7 times with 1-mL portions of 0.1 M NH_4CO_3 to remove excess radiolabeled AdoMet. The tRNA was then eluted with 1 mL of a solution of 0.05 N NaOH and 1 M KCl, the resin was pelleted by centrifugation, and 0.9 mL of the supernatant was counted in 10 mL of Aquasol-2. This procedure yielded 90% of the labeled tRNA in the final supernatant.

Enzyme Purification. *E. coli* GB1-5-39/pTN102 overproduces tRNA (Ura-5-)methyltransferase 20–40-fold and was used to pepare enzyme by a slight modification of a reported procedure (Greenberg & Dudock, 1980). Cells were grown in the rich nutrient medium as described above, chilled just as cell growth began to slow from log phase (A_{600} = ca. 4.5), and centrifuged. Cells harvested at higher density yielded less enzyme per gram of cells and lower initial specific activity. Cell lysis, removal of nucleic acids by poly(ethylenimine) titration, ammonium sulfate precipitation, dialysis, and phosphocellulose chromatography were performed as described (Greenberg & Dudock, 1980). Instead of chromatography of the enzyme on Blue Sepharose and elution by tRNA, the final step was chromatography on DEAE-cellulose at pH 6.5, 4 °C, as follows. A solution of 80 mL containing 2.5 × 10^4 units of tRNA (Ura-5-)methyltransferase activity (specific activity 510 units/mg; A_{280} = 0.11) eluted from phosphocellulose was equilibrated by dialysis at 4 °C against a pH 6.5 solution containing 20 mM Pipes, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 20% (w/v) glycerol (starting buffer). The dialyzate was applied to a 10-cm^3 column of DEAE-cellulose previously equilibrated with starting buffer. The column was washed with additional starting buffer until the effluent A_{280} decreased to 0.01; enzyme was eluted with a linear gradient of 50 mL each of starting buffer and 0.2 M KCl in starting buffer. Fractions of 2 mL were collected over the gradient elution, and 60% of the applied activity eluted in fractions 12–18. The two peak fraction containing 30% of the applied activity had a specific activity of 3400 units/mg and were ~80% pure as by densitometric scanning of Coomassie Blue stained SDS–polyacrylamide gels. Greenberg and Dudock report the molecular weight of the methylase (Greenberg & Dudock, 1980) to be 42 000; we observed M_r 41 000.

Inhibitor Studies. The effect of FUra-tRNA on tRNA (Ura-5-)methyltransferase activity was determined by preincubating the enzyme with FUra-tRNA in the presence and absence of AdoMet. Methyltransferase (0.4 μg/mL) was incubated in preincubation buffer (50 mM Tes, pH 6.6, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM dithiothreitol, and 40 mM ammonium acetate) with (a) 7.7 μM m^{5}U-deficient tRNA and 125 μM AdoMet, (b) 7.7 μM FUra-tRNA and 125 μM AdoMet, or (c) 7.7 μM FUra-tRNA. Aliquots of 15 μL were removed at timed intervals and diluted into 500 μL (~33-fold) of assay buffer containing 125 μM AdoMet and 0.6 μM [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA, and the residual methyltransferase activity was measured by using the tritium release assay described above.

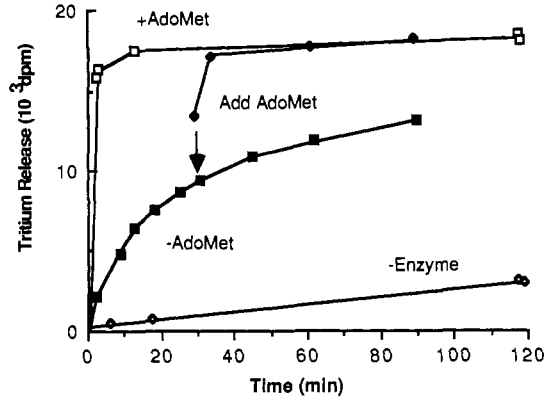


FIGURE 1: Appearance of tritium in solvent water from [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA (0.6 μM, 8.7 Ci/mmol) catalyzed by tRNA (Ura-5-)methyltransferase. In addition to the labeled tRNA, reactions contained (□) 0.3 μg of enzyme/mL and 125 μM AdoMet, (■) 2.9 μg of enzyme only, or (◇) no enzyme. Aliquots of 100 μL were assayed for solvent tritium by the charcoal method. At the time indicated by the arrow, a portion of the reaction (◆) with 2.9 μg of enzyme/mL without AdoMet was made 125 μM in AdoMet.

SDS–PAGE analysis of complexes between tRNA (Ura-5-)methyltransferase and FUra-tRNA were performed as follows. A solution (80 μL) containing enzyme (82 units, 24 μg), 11.5 μM FUra-tRNA, and 125 μM [^{3}H]methyl-AdoMet (0.5 Ci/mmol) or 1.25 mM unlabeled AdoMet in preincubation buffer was incubated at 37 °C for 18 h. At this time, half of each sample was mixed with 5 μL (22 units) of a 1 mg/mL solution of pancreatic RNase (Worthington; 4340 units/mg), and the incubations were continued for an additional 2.5 h. The following reactions were performed in parallel: enzyme + 11.5 mM FUra-tRNA; enzyme + 11.5 μM m^{5}U-deficient tRNA + 1.25 mM AdoMet; and enzyme + 11.5 μM FUra-tRNA + 5.6 mM AdoMDB. Samples were denatured by boiling in 3% SDS for 3 min and electrophoresed in a 10% polyacrylamide gel (Laemmli, 1970). Samples containing tritium were precipitated at 4 °C by addition of an equal volume of cold 10% trichloroacetic acid, centrifuging, and washing the pellets with ice-cold water to remove excess radioactive AdoMet prior to electrophoresis. Lanes containing tritium-labeled samples were fractionated into 3-mL segments. Each segment was treated with 1 mL of Protosol (New England Nuclear) at 70–80 °C for 1 h, neutralized with 0.1 mL of glacial acetic acid, and counted in 10 mL of ACS (Amersham).

RESULTS

Methylation and Tritium Release. tRNA (Ura-5-)-methyltransferase catalyzes release of tritium from [5-^{3}H]-Urd-labeled m^{5}U-deficient tRNA accompanying methylation by AdoMet (Figure 1). The amount of tritium released in this reaction is stoichiometric with the amount of methylation that occurs in the AdoMet-dependent reaction, as demonstrated by the following results.

HPLC analysis of enzyme digests of tRNA revealed the presence of 1 mol of m^{5}U per mole of tRNA in wild-type *E. coli* tRNA but less than 0.02 mole per mole of tRNA in the methylase-deficient *E. coli* GB1-5-39. Treatment of m^{5}U-deficient tRNA with excess [^{3}H]methyl-AdoMet and enzyme resulted in the incorporation of 0.94 mol of [^{3}H]methyl groups per mole of tRNA. This is accounted for by methylation of

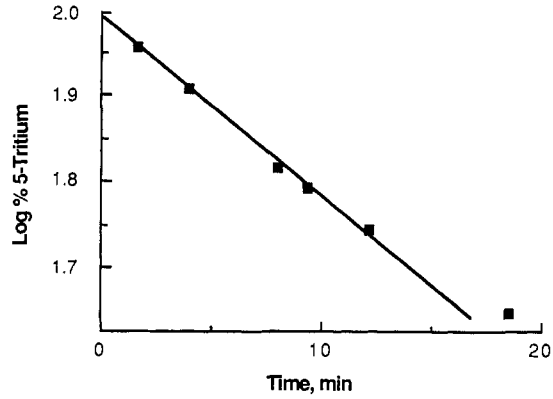


FIGURE 2: First-order plot of data for tritium exchange from [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA (0.6 μM, 8.7 Ci/mmol) into solvent water catalyzed by tRNA (Ura-5-)methyltransferase (5.9 μg/mL) in the absence of AdoMet. Aliquots of 100 μL were assayed for tritium exchange by the charcoal adsorption method.

a single Urd residue per tRNA molecule to generate the m^{5}U residue in the TΨC loop.

HPLC analysis demonstrated an average of 38 mol of pyrimidine (Urd + Cyd) residues per mole of tRNA in bulk tRNA isolated from *E. coli* GB1-5-39. These Urd and Cyd residues should be equally labeled when cells are grown in the presence of [5-^{3}H]Urd (Cortese et al., 1974). The maximum tritium release from [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA catalyzed by the methyltransferase in the presence of excess AdoMet is about 2.5% of the total tritium present in the tRNA. This value indicates that approximately 1 out of 40 labeled residues per tRNA is susceptible to the action of the methyltransferase, in agreement with the amount of thymine found in tRNA. The stoichiometry of tritium release therefore indicates that the methyltransferase modifies 1 pyrimidine residue per tRNA, accounted for by the Urd precursor to m^{5}U in the TΨC loop.

In order to determine the relative rates of methylation and tritium release under identical conditions and substrate concentrations, a double-label experiment was performed with [^{14}C]methyl-AdoMet and [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA. The results of this experiment (Figure 3) indicate that methylation and tritium release in the presence of AdoMet occur with essentially identical kinetics and stoichiometry.

AdoMet-Independent Tritium Exchange. In addition to the obligatory tritium release accompanying methylation, the methyltransferase also catalyzes an exchange reaction between the tritium of [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA and protons of water in the absence of AdoMet (Figure 1). If methylation and tritium exchange occurs at the same Urd residue(s), the composite of partial reactions should not exceed the total methylation sites. After substantial partial exchange (50%) had occurred in a reaction containing methyltransferase and [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA, AdoMet was added. The final amount of tritium release after methylation was identical with that found in a control reaction in which AdoMet was present at the outset (Figure 1). This identity indicates that the AdoMet-independent exchange reaction catalyzed by the methyltransferase occurs at the same Urd residue in tRNA as the normal methylation reaction.

tRNA (Ura-5-)methyltransferase catalyzed tritium exchange is expected to exhibit first-order kinetics. A first-order plot of the data from the AdoMet-independent exchange reaction (Figure 2) is linear up to the release of at least half of the exchangeable tritium. The first-order rate constants for this exchange reaction were linear with enzyme concentration over a 20-fold range. The value of the first-order rate constant

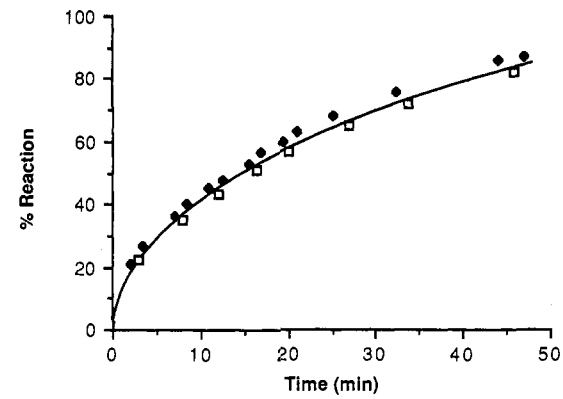


FIGURE 3: Tritium release (□) and [^{14}C]methyl incorporation (♦) from [^{14}C]methyl-AdoMet into m^{5}U-deficient tRNA. Reactions were monitored as described under Materials and Methods.

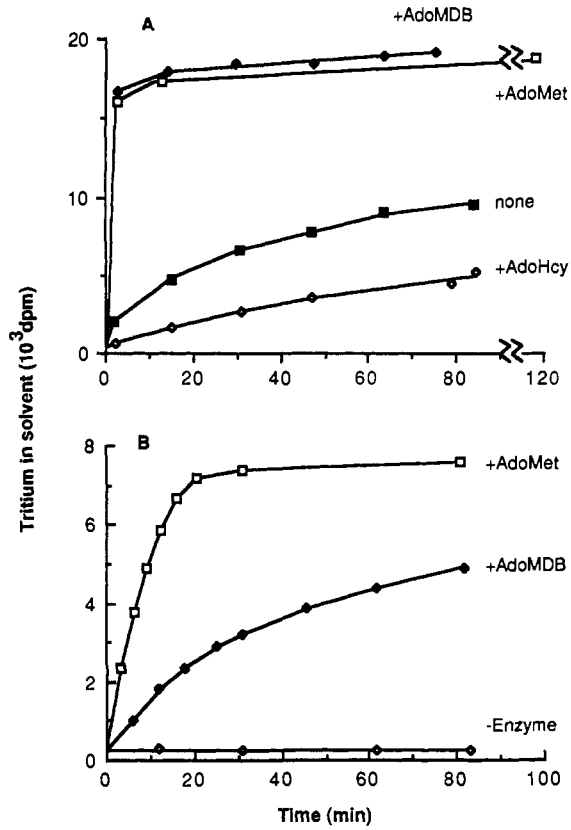


FIGURE 4: Effects of AdoHcy and AdoMDB on rate of tritium exchange/release reaction. Reactions contained the following: (A) 0.6 μM [5-^{3}H]Urd-labeled m^{5}U-deficient tRNA (8.7 Ci/mmol) and (■) 0.95 μg of enzyme/mL, (♦) 0.95 μg of enzyme/mL and 9 mM AdoMDB, (◊) 0.95 μg of enzyme/mL and 125 μM AdoHcy, or (□) 0.3 μg of enzyme/mL and 125 μM AdoMet; (B) 16 μM [5-^{3}H]-Urd-labeled m^{5}U-deficient tRNA (0.21 Ci/mmol) and (♦) 6.8 μg of enzyme/mL and 9 mM AdoMDB, (□) 6.8 μg of enzyme/mL and 125 μM AdoMet, or (◊) no addition. In both (A) and (B), aliquots of 100 μL were assayed for solvent tritium by the charcoal adsorption method.

for the AdoMet-independent exchange reaction and also the value of the initial velocity of tritium release accompanying methylation were independent of tRNA concentration in the range 0.1–3.0 μM (data not shown).

In the absence of AdoMet, the rate of the methyltransferase-catalyzed tritium exchange reaction is 4 nmol min^{-1} mg^{-1}; this is about 1% of the initial velocity of methyltransferase-catayzed methylation/tritium release in the presence of saturating (125 μM) AdoMet (320 nmol min^{-1} mg^{-1}). The effects of AdoHcy and AdoMDB on the rate of

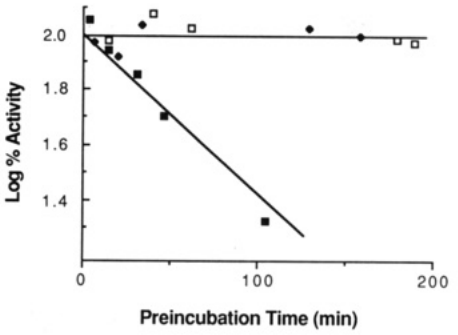


FIGURE 5: Inactivation of tRNA (Ura-5-)methyltransferase by FUra-tRNA plus AdoMet. The enzyme (0.4 μg/mL) was preincubated at 37 °C in preincubation buffer with (□) 7.7 μM m^{5}U-deficient tRNA and 125 μM AdoMet, (♦) 7.7 μM FUra-tRNA, or (■) 7.7 μM FUra-tRNA and 125 μM AdoMet. Aliquots of 15 μL were diluted 33-fold into an assay cocktail, and the residual activity was measured by the tritium-release assay as described under Materials and Methods. The values shown are percentages of the average value of the activity measurements for the enzyme incubated with m^{5}U-deficient tRNA and AdoMet.

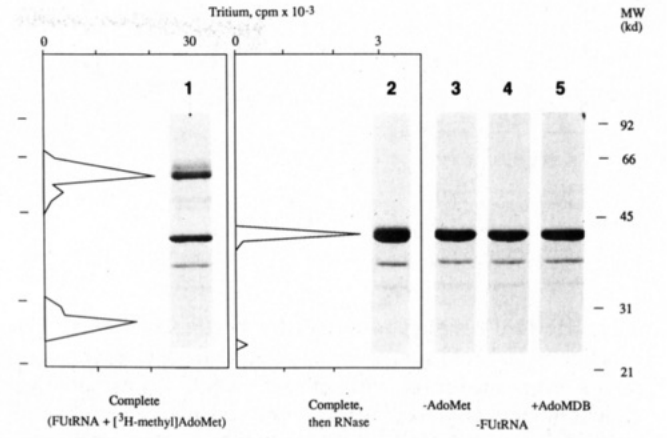


FIGURE 6: Formation of covalent complex between tRNA (Ura-5-)methyltransferase, FUra-tRNA, and AdoMet. Samples (prepared and electrophoresed as described under Materials and Methods) were as follows: Lane 1, enzyme + FUra-tRNA + [^{3}H]methyl-AdoMet; lane 2, enzyme + FUra-tRNA + [^{3}H]methyl-AdoMet, then RNase; lane 3, enzyme + FUra-tRNA; lane 4, enzyme + m^{5}U-deficient tRNA + AdoMet; lane 5, enzyme + FUra-tRNA + AdoMDB. Shown for lanes 1 and 2 are plots of the counts per minute (cpm) of tritium measured by scintillation counting of the fractionated lanes. Gels were stained for protein with Coomassie Blue. Molecular weight standards used (not shown) were phosphorylase B (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), and soybean trypsin inhibitor (M_r 21 500).

the exchange reaction are shown in Figure 4. The rate of exchange is decreased 2–3-fold in the presence of 125 μM AdoHcy, a concentration which is 30 times the reported K_i value for AdoHcy inhibition of the methylation reaction (Shugart, 1978). In the presence of the AdoMet analogue AdoMDB (9 mM), the rate of exchange *increases* 9-fold (37 μmol min^{-1} mg^{-1}). At this concentration of AdoMDB, the methylation activity of the methyltransferase, as assayed by [^{3}H]methyl incorporation with 1.7 μM [^{3}H]methyl-AdoMet, is inhibited by 72% (data not shown).

To ascertain whether AdoMDB might act as a methyl donor, the following experiment was performed. The reaction containing AdoMDB, [5-^{3}H]Urd-tRNA, and enzyme was allowed to proceed for 90 min, at which time 65% of the tritium was released into solvent (Figure 4B). A portion of the reaction mixture was then mixed with an excess of [^{14}C]methyl-AdoMet (final concentration 66 μM), and the stoichiometry of methyl groups per tRNA was determined 60 and 90 min later. The observed stoichiometry was identical with that found after in vitro methylation of otherwise untreated m^{5}U-deficient tRNA, i.e., 0.9 [^{14}C]methyl group per tRNA. Thus, the effect of AdoMDB in accelerating the methyltransferase-catalyzed tritium exchange reaction is not due to AdoMDB serving as a methyl donor.

The [5-^{3}H]Urd residue at position 54 of m^{5}U-deficient tRNA does not undergo rapid exchange in the absence of enzyme. The rate of chemical exchange of tritium from [^{3}H]Urd-labeled m^{5}U-deficient tRNA is identical with the rate of exchange from labeled tRNA after it has been fully methylated in vitro by the methyltransferase. Both have first-order rate constants for exchange at 30 °C, pH 8.4, of 3×10^{-4} h^{-1} (data not shown), about 10-fold higher than the values reported by Shoemaker and Schimmel (1977) for chemical exchange of tritium from C-5 of labeled pyrimidine in *E. coli* tRNA^{Tyr} at 30 °C, pH 6.5; the differing pH values used are probably responsible for the difference in the chemical exchange rates (Santi & Brewer, 1973).

Inhibition by FUra-tRNA. Incubation of tRNA (Ura-5-)methyltransferase at 37 °C, pH 6.6, with 7.7 μM FUra-tRNA and 125 μM AdoMet resulted in a time-dependent inactivation of enzyme activity measured in subsequent AdoMet-dependent tritium-release assays. A first-order plot of the inactivation data from such an experiment is shown in Figure 5. Under these conditions, the inactivation proceeded with $k = 0.014$ min^{-1}. tRNA (Ura-5-)methyltransferase incubated with FUra-tRNA and AdoMet was totally inhibited after 20 h, but no decrease was observed in the activity of methyltransferase incubated under identical conditions with 7.7 μM m^{5}U-deficient tRNA and 125 μM AdoMet, or with 7.7 μM FUra-tRNA in the absence of AdoMet. Hence, inactivation of the enzyme by FUra-tRNA requires AdoMet.

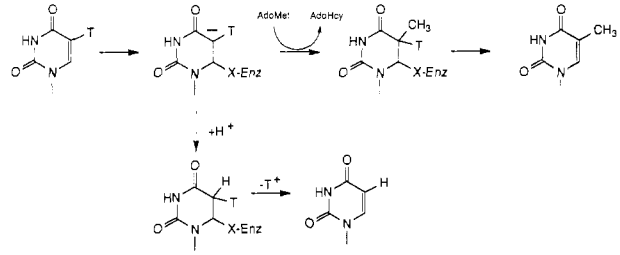
Incubation of purified tRNA (Ura-5-)methyltransferase with FUra-tRNA and AdoMet led to a 70% decrease in the intensity of the protein band at M_r 41 000 on SDS–PAGE, which corresponds to the enzyme (Figure 6, lane 1). This was accompanied by the appearance of several protein bands centered around M_r 61 000 which were labeled by the tritium from [^{3}H]methyl-AdoMet. The additional tritium-labeled material at apparent M_r 27 000 is [^{3}H]methyl-labeled tRNA. The increase in the apparent size of tRNA (Ura-5-)methyltransferase was not observed when the enzyme was exposed to FUra-tRNA alone (Figure 6, lane 3), to m^{5}U-deficient tRNA (lane 4), or to FUra-tRNA plus the AdoMet analogue AdoMDB (lane 5). RNase treatment of enzyme previously preincubated with FUra-tRNA and AdoMet (lane 1) reconverted the higher molecular weight material to a band that migrates almost identically with that of the untreated tRNA (Ura-5-)methyltransferase (lane 2) and was still tritium-labeled. In the experiment shown, the yield of tritium-labeled material after RNase digestion was significantly less than the yield in the undigested sample, but subsequent experiments gave approximately identical amounts of tritium incorporation before and after RNase digestion (data not shown).

Sulfhydryl Requirement. To test for a sulfhydryl group essential to catalysis by tRNA (Ura-5-)methyltransferase, the enzyme was assayed by using the tritium release assay in the presence of the *p*-(chloromercuri)benzoate. The methyltransferase had no activity in the presence of 2.5 mM *p*-(chloromercuri)benzoate; the inhibition was completely reversed by the subsequent addition of 5 mM dithiothreitol.

Discussion

We have shown that, in the absence of AdoMet, tRNA (Ura-5-)methyltransferase catalyzes exchange of the 5-H of

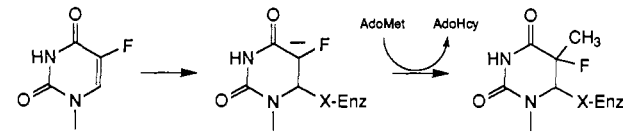
Scheme I



substrate Urd residues in tRNA for protons of water. The reaction is 100-fold slower than the methylation reaction and shows rate saturation with excess tRNA. The identical stoichiometry observed for tritium release accompanying methylation of [5-^{3}H]Urd-labeled tRNA by AdoMet and tritium exchange in the absence of AdoMet indicates that these two reactions occur at identical Urd residues on the tRNA, the Urd residue that is the precursor to the m^5U in the TΨC loop. The effects of analogues of AdoMet (AdoHcy, AdoMDB) on the rate of the exchange reaction is further evidence for the similar nature of these two reactions. At saturating concentrations, AdoHcy inhibits both the exchange and methylation reactions, as expected of product inhibition at the active site. [AdoHcy suppresses a similar exchange reaction catalyzed by the DNA (Cyt-5-)methyltransferase *Hha*I (Wu & Santi, 1987).] At high concentrations, the nitrogen analogue of AdoMet, AdoMDB, also inhibits methylation of tRNA, presumably by competing with AdoMet. However, AdoMDB *increases* the rate of the exchange reaction catalyzed by tRNA (Ura-5-)methyltransferase by an order of magnitude. The tertiary amine group of AdoMDB should be protonated at neutral pH and therefore charged identically to the sulfonium group of AdoMet which it replaces. AdoHcy lacks this charge as well as the methyl group. Acceleration of the exchange reaction by AdoMDB suggests that binding of this close analogue of AdoMet may promote a catalytically favorable conformational change of the enzyme.

Scheme I shows chemically reasonable mechanisms for the AdoMet-dependent and AdoMet-independent tRNA (Ura-5-)methyltransferase catalyzed release of tritium from m^5U-deficient [5-^{3}H]Urd-tRNA into solvent. The evidence for 5,6-saturated pyrimidine intermediates in similar exchange reactions in nonenzymatic systems has been reviewed (Pogolotti & Santi, 1977). Analogous enzyme-catalyzed exchange of tritium from 5-^{3}H substrates with solvent protons has been observed with several enzymes (Pogolotti et al., 1979; Kunitani & Santi, 1980; Wu & Santi, 1987). In fact, the correlation between enzyme-catalyzed 5-H exchange and the formation of 5,6-dihydropyrimidine intermediates is sufficiently strong that the observation of the exchange reaction is itself good evidence for the mechanism. Enzyme-catalyzed formation of 5,6-dihydropyrimidine intermediates involves reversible Michael addition of an enzymic nucleophile to carbon 6 of the pyrimidine, generating an anion equivalent at carbon 5. In the case of tRNA (Ura-5-)methyltransferase, the anion equivalent at carbon 5 would be methylated in the presence of AdoMet and tritium release from carbon 5 would accompany β-elimination of the enzymic nucleophile. In analogy with TS, in the absence of AdoMet exchange of tritium at carbon 5 could proceed by random, nonstereospecific protonation and deprotonation at carbon 5; alternatively, it could occur by stereospecific addition of a proton at carbon 5 to that face of the heterocycle that normally accepts a methyl group, followed by stereospecific removal of tritium from the opposite face and expulsion of the enzymic nucleophile. The results

Scheme II



presented here cannot distinguish between these two mechanisms.

The inactivation of tRNA (Ura-5-)methyltransferase by FUra-tRNA in the presence of AdoMet provides further evidence for a covalent intermediate in catalysis. The proposed mechanism for this inactivation is shown in Scheme II. Here, an enzymic nucleophile adds to the 6-position of FUra within the substrate site of tRNA to provide an adduct which is subsequently trapped by AdoMet-dependent methylation at carbon 5. The ternary covalent complex is analogous to a steady-state intermediate proposed in the normal enzymic reaction (Scheme I) and is similar in structure to that of the covalent complex formed between TS, methylenetetrahydrofolate, and the substrate analogue 5-FdUMP (Santi & Danenberg, 1984). The methylated FUra-tRNA–enzyme covalent complex cannot break down to give product and regenerate the free enzyme, because the hydrogen at carbon 5 of the normal substrate has been replaced by fluorine; similarly, reversal of the reaction is highly unfavorable since it would require the energetically unfavorable cleavage of a carbon–carbon bond at the 5-position. Thus, we conclude that formation of the methylated FUra-tRNA–enzyme complex is irreversible.

SDS–PAGE analysis of tRNA (Ura-5-)methyltransferase before and after inactivation by FUra-tRNA and [^{3}H]methyl-AdoMet supports the structure of the covalent complex proposed in Scheme II. The inactivated enzyme has a decreased electrophoretic mobility consistent with its proposed covalent link to tRNA and is labeled by tritium derived from the methyl group of [^{3}H]methyl-AdoMet. Treatment of the enzyme–tRNA complex with RNase results in an electrophoretic shift to a mobility almost identical with that of the enzyme before inactivation, but the inactivated enzyme is still labeled with tritium of the methyl group. We conclude that RNase removes most of the RNA from the complex but leaves a [^{3}H]methyl-FUrd residue covalently bound to the protein. In the absence of AdoMet, or in the presence of AdoMet analogues such as AdoMDB, which cannot donate a methyl group, isolable denatured complexes are not observed. It is evident from these results that FUra-tRNA is a potent and irreversible mechanism-based inhibitor of tRNA (Ura-5-)-methyltransferase.

The nucleophile in TS-catalyzed reactions and in covalent complex formation with FdUMP has been demonstrated to be a Cys residue located in a conserved Pro-Cys sequence (Santi & Danenberg, 1984); it has also been proposed that Cys residues of *Hha*I and other DNA (Cyt-5-)methyltransferases have a similar role (Wu & Santi, 1987). The observation that tRNA (Ura-5-)methyltransferase activity is inhibited by *p*-(chloromercuri)benzoate, and the precedent of these related reactions, leads us to propose that the nucleophile involved in catalysis by this methyltransferase is also a cysteine residue.

The tRNA (Ura-5-)methyltransferase is a member of a growing class of enzymes that catalyzes one-carbon transfer reactions at the 5-position of pyrimidines by nucleophilic catalysis as depicted in Scheme I. These include thymidylate synthase (Santi & Danenberg, 1984), dUMP hyhdroxymethylase (Kunitani & Santi, 1980), dCMP hydroxymethylase

(Yeh & Greenberg, 1967), and DNA (Cyt-5-)methyltransferase (Wu & Santi, 1987). At the present time, this group of enzymes is sufficiently large and diverse that it is reasonable to speculate that all such electrophilic substitution reactions share salient features of the general mechanism.

tRNA (Ura-5-)methyltransferase also represents a protein that forms a covalent bond with carbon 6 of a pyrimidine residue in a nucleic acid. The first such proteins were reported by Schimmel and colleagues, who showed that aminoacyl-tRNA synthetases catalyze exchange reactions between solvent and hydrogen at carbon 5 of Urd residue 8 in cognate tRNAs (Shoemaker & Schimmel, 1977) and undergo putative covalent bond formation with the 6-position of 5-bromouridine (Starzyk et al., 1982). Bacteriophage R17 coat protein has also been suggested to undergo similar covalent bond formation with 5-bromopyrimidine nucleosides and a 21-nucleotide RNA hairpin containing a specific binding site for that protein (Romaniuk & Uhlenbeck, 1985). A methyl donor independent tritium exchange reaction, similar to that reported here, was observed (Nagle, 1980) with the methylenetetrahydrofolate-dependent tRNA (Ura-5-)methyltransferase from *Streptococcus faecalis* and suggests similar covalent interactions. DNA (Cyt-5-)methyltransferases have recently been shown to function catalytically by forming transient Michael adducts between an enzymic nucleophile and carbon 6 of the pyrimidine (Wu & Santi, 1987). DNA containing 5-azacytosine residues or FdC is a potent inhibitor of both prokaryotic (Friedman, 1979) and eukaryotic (Taylor & Jones, 1982) DNA (Cyt-5-)methyltransferases, and the mechanism has been shown to involve covalent bond formation between C-6 and an enzymic nucleophile (Santi et al., 1984; Friedman, 1985). Thus, formation of reversible Michael adducts between a nucleophile on a protein and carbon 6 of a pyrimidine residue in nucleic acids seems to be a general feature of some protein–polynucleotide interactions. It appears to be involved in simple binding reactions in some cases and to play a central catalytic role in others.

Finally, the inactivation of tRNA (Ura-5-)methyltransferase by covalent complex formation with FUra-tRNA may be directly relevant to the action of FUra. In addition to inhibition of TS by 5-FdUMP, the cytotoxic action of FUra is known to involve incorporation into RNA; the subsequent events which lead to significant biological effects remain controversial (Heidelberger et al., 1983; Chabner, 1984). From the present work, we can propose mechanisms that may contribute to the "RNA effect" of FUra. First, FUra-tRNA inhibition of tRNA (Ura-5-)methyltransferase will deplete the m^5U in the TΨC loop of tRNA. However, hypomethylation at this site of tRNA in itself cannot be a major cell deficit since tRNA (Ura-5-)methyltransferase deficient mutants of *E. coli* (Bjork & Neidhardt, 1975) and yeast (Hopper et al., 1982) are healthy; further, FUra-tRNA is functional in protein synthesis (Ramberg et al., 1978). In addition to m^5U of the TΨC loop of tRNA, other modified pyrimidines of RNA are probably also formed by a mechanism involving transient covalent adducts between specific enzymes and the substrate pyrimidine residue (Santi et al., 1978). These include m^5U of other RNAs, Ψrd, m^5C, and several other 5-alkylpyrimidines. Further, such modifications may involve tRNA, ribosomal RNA (Bjork, 1984), and, in eukaryotic cells, the small nuclear RNAs (Busch et al., 1982). Hence, FUra could interfere with a variety of important enzymes and cellular processes, and hypomodification of one or more sites of one or more RNAs is an attractive contender for the RNA effect of FUra. Alternatively, or concomitantly, covalent FUra-RNA–enzyme complexes could play a direct role in the effects of FUra.

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Kinetics and Thermodynamics of the Interaction of 5-Fluoro-2′-deoxyuridylate with Thymidylate Synthase†

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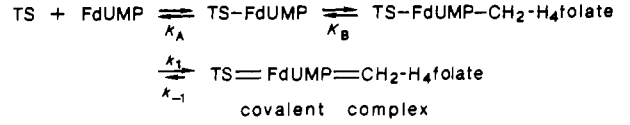
ABSTRACT: Thymidylate synthase (TS), 5-fluorodeoxyuridylate (FdUMP), and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) form a covalent complex in which a Cys thiol of TS is attached to the 6-position of FdUMP and the one-carbon unit of the cofactor is attached to the 5-position. The kinetics of formation of this covalent complex have been determined at several temperatures by semirapid quench methods. Together with previously reported data the results permit calculation of every rate and equilibrium constant in the interaction. Conversion of the noncovalent ternary complex to the corresponding covalent complex proceeds at a rate of 0.6 s^{-1} at 25 °C, and the dissociation constant for loss of CH_2-H_4folate from the noncovalent ternary complex is ~1 μM. Activation parameters for the formation of the covalent complex were shown to be E_a = 20 kcal/mol, $\Delta G^{\ddagger}$ = 17.9 kcal/mol, $\Delta H^{\ddagger}$ = 19.3 kcal/mol, and $\Delta S^{\ddagger}$ = 0.005 kcal/(mol·deg). The equilibrium constant between the noncovalent and covalent ternary complexes is ~2 × 10^4, and the overall dissociation constant of CH_2-H_4folate from the covalent complex is ~10^{-11} M. The conversion of the noncovalent ternary complex to the covalent adduct is about 12-fold slower than k_{cat} in the normal enzymic reaction. However, because the dissociation constant for CH_2-H_4folate from the noncovalent ternary complex is about 10-fold lower than that from the TS–dUMP–CH_2-H_4folate Michaelis complex, the terms corresponding to k_{cat}/K_m are nearly equal. We propose that some of the intrinsic binding energy of CH_2-H_4folate may be used to facilitate formation of a 5-iminium ion intermediate. We suggest that iminium ion formation is catalyzed by (a) general-acid catalysis at N-10 of CH_2-H_4folate and (b) enzyme-induced perturbations of the five-membered ring of the cofactor within the noncovalent TS–FdUMP–CH_2-H_4folate complex. The latter may involve hydrogen bonding of the enzyme general-acid catalyst to N-10, perturbation of the *p*-aminobenzoic acid moiety of the cofactor, and strain on the five-membered ring of the cofactor.

5-Fluoro-2′-deoxyuridylate (FdUMP)[1] behaves as a mechanism-based inhibitor in the thymidylate synthase (TS) reaction. In the presence of TS, CH_2-H_4folate, and FdUMP, enzymic conversions occur up to the step normally associated with methyl transfer to the pyrimidine heterocycle, thereby trapping a covalent TS=FdUMP=CH_2-H_4folate complex. The covalent complex is an analogue of a steady-state intermediate of the normal enzymic reaction and has a Cys thiol of TS attached to the 6-position of FdUMP and the one-carbon unit of the cofactor attached to the 5-position of the nucleotide (Santi & Danenberg, 1984). Because of its importance in chemotherapy of neoplastic disease, the interaction of FdUMP with TS has been extensively studied. Furthermore, since the interaction mimics several steps in the normal enzymic reaction, it provides an approach toward studying individual steps of the reaction that are otherwise inaccessible. Now that the

Scheme I



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[1] Abbreviations: TS, thymidylate synthase; FdUMP, 5-fluoro-2′-deoxyuridylate; CH_2-H_4folate, (6*R*)-L-5,10-methylenetetrahydrofolate; TS–FdUMP–CH_2-H_4folate, noncovalent ternary complex; TS=FdUMP=CH_2-H_4folate, covalent ternary complex; NMM, *N*-methylmorpholine; DTT, dithiothreitol; PABA, *p*-aminobenzoic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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