Identification of the Catalytic Nucleophile of tRNA (m^5U54)Methyltransferase†

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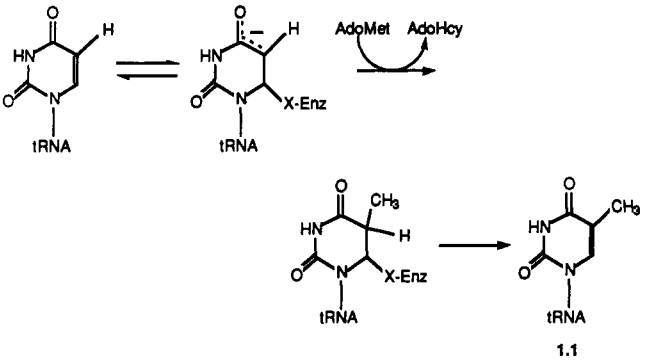
ABSTRACT: A covalent complex between tRNA (m^5U54)methyltransferase, 5-fluorouridine $tRNA^{Phe}$, and *S*-adenosyl-L-[*methyl*-^{3}H]methionine was formed in vitro and purified. Previously, it was shown that in this complex the 6-position of fluorouridine-54 is covalently linked to a catalytic nucleophile and the 5-position is bound to the transferred methyl group of AdoMet [Santi, D. V., & Hardy, L. W. (1987) *Biochemistry 26*, 8599–8606]. Proteolysis of the complex generated a [^{3}H]methyl-FUtRNA-bound peptide, which was purified by 7 M urea–15% polyacrylamide gel electrophoresis. The peptide component of the complex was sequenced by gas-phase Edman degradation and found to contain two cysteines. The tritium was shown to be associated with Cys 324 of the methyltransferase, which unequivocally identifies this residue as the catalytic nucleophile.

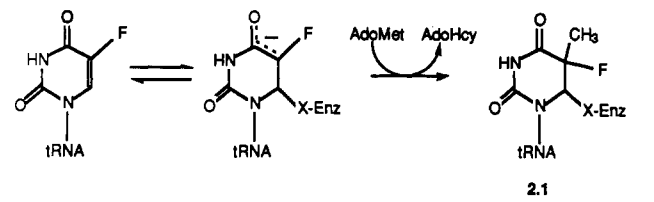
tRNA (m^5U54)methyltransferase (RUMT;[1] EC 2.1.1.35) catalyzes the AdoMet-dependent methylation of uridine 54 (U54) in the TΨC loop of all eubacterial and most eukaryotic tRNAs (Sprinzl et al., 1985). As shown in Scheme I, the reaction is believed to occur by (i) Michael addition of a nucleophilic group of the enzyme to the 6-carbon of U54, followed by (ii) methyl transfer from AdoMet to the 5-position of U54, (iii) 5-proton abstraction, and finally, (iv) β-elimination of the enzyme to yield ribothymidine (**1.1**). This mechanism is supported by the experimental observation that RUMT catalyzes proton exchange between 5-[^{3}H]uridine-tRNA and solvent water in the absence of AdoMet. Also, when fluorine is substituted for hydrogen at the 6-position of uridine, RUMT forms an irreversible, covalent complex with the FUtRNA in the presence of AdoMet (Santi & Hardy, 1987). Because of the stability of the carbon–fluorine bond, the Michael adduct is trapped as the FUtRNA-CH_3–enzyme complex (**2.1**, Scheme II). RUMT is inactivated by the thiol reagent *p*-(chloromercuri)benzoate, and it has been suggested that the catalytic nucleophile may be a cysteine.

A paradigm for the mechanism of RUMT is the thymidylate synthase catalyzed reductive methylation of dUMP, where Michael addition of a cysteine nucleophile across the C5–C6 double bond of the pyrimidine has been unequivocally established [see Santi and Danenberg (1984)]. Addition of an enzyme nucleophile to the 6-carbon of deoxyribopyrimidines also occurs in reactions catalyzed by dUMP hydroxymethylase (Kunitani & Santi, 1980), dCMP hydroxymethylase (Basak et al., 1988), and DNA (Cyt-5-)methyltransferase (Osterman et al., 1988; Wu & Santi, 1987). Further, Michael addition to pyrimidines of RNA has been proposed to occur in complexes between the aminoacyl-tRNA synthetases and tRNA (Starzyk et al., 1982), the R17 coat protein and bacteriophage R17 replicase gene (Romaniuk & Uhlenbeck, 1985), and a 55-kDa protein and RNA in the 5′-noncoding region of poliovirus (Najita & Sarnow, 1990). These suggest that Michael adduct formation may be a common mechanism for protein–RNA interactions. In all of the aforementioned additions to pyrimidines of RNA, a cysteine residue of the protein has been implicated as the nucleophile, but in no case has this been proved.

Scheme I



Scheme II



Since RUMT forms an isolable covalent complex with FUtRNA and a methyl group derived from AdoMet, it provides a model system for detailed study of protein-mediated Michael addition to a pyrimidine of RNA. The obligatory first step of the reaction can be established by elucidation of the amino acid residue that is covalently bound to FUtRNA.

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[1] Abbreviations: RUMT, tRNA (m^5U54)methyltransferase; FUtRNA, 5-fluorouracil yeast $tRNA^{Phe}$; Tris, tris(hydroxymethyl)aminomethane; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; PVDF, poly(vinylidene difluoride); NTPs, ribonucleotide triphosphates; LEP, lysylendopeptidase [*Achromobacter* protease I (EC 3.4.21.50)]; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; ATZ, anilinothiazolinone; PTH, phenylthiohydantoin; PAGE, polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; FPLC, fast protein liquid chromatography; DITC, 1,4-phenylene diisothiocyanate; V-8 protease, staphylococcal protease (strain V-8) (EC 3.4.21.19); XC, xylene cyanole; i.d., internal diameter; Gdn-HCl, guanidine hydrochloride; EtdBr, ethidium bromide.

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In this paper we report the isolation and primary sequence of a fragment of RUMT which remains covalently bound to FUtRNA, following proteolysis of the covalent complex. We have also identified Cys 324 of RUMT as the nucleophile that is covalently bound to the ribopyrimidine.

Materials and Methods

Acrylamide, bis(acrylamide), and CAPS were from Bio-Rad. AdoMet was from Boehringer Mannheim. Ribonucleotide triphosphates were from Pharmacia. 5-Fluorouridine triphosphate was from Sierra Bioresearch. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (79 Ci/mmol) and [^{14}C]iodoacetamide (59 Ci/mol) were from Amersham. Aquasol II was from Du Pont. ProBlott PVDF membrane was from Applied Biosystems. LEP was from Wako. T-7 RNA polymerase was prepared as described (Davanloo et al., 1984). Unless otherwise specified, DEAE-cellulose was DE-52 from Whatman. DITC Sequalon membranes and associated reagents were from Millipore. Peptide sequencing was performed on an Applied Biosystems 470A gas-phase protein sequencer with an on-line 120A PTH analyzer at the Biomolecular Resource Center, University of California, San Francisco. EtOH precipitations were performed by addition of 2.5 volumes of cold 95% EtOH, followed by cooling at −20 °C overnight or at −80 °C for 1 h.

Purification of RUMT. RUMT was purified to 800 units/mg (ca. 50% pure) as described (Gu & Santi, 1990), except the final DEAE purification step was omitted. One unit is the amount of enzyme that transfers 1 pmol of methyl groups to U54 methyl-deficient tRNA per minute at 30 °C under standard assay conditions (Santi & Hardy, 1987).

Preparation and Purification of FUtRNA^{Phe}. FUtRNA^{Phe} was prepared by in vitro transcription as described (Sampson & Uhlenbeck, 1988), using 4 mM NTPs and replacing UTP with FUTP. After phenol/chloroform extraction and EtOH precipitation, the RNA pellet was dissolved in water and applied to a Nucleogen 4000-10 DEAE column (1 × 12 cm), equilibrated with 20 mM Tris-HCl, pH 7.6, containing 0.1 M NaCl (buffer A). The column was washed with buffer A for 5 min at 0.5 mL/min, followed by a linear ramp to 40% buffer B (20 mM Tris-HCl, pH 7.6, 1 M NaCl) over 5 min; from 40% to 65% B over 40 min; from 65% to 100% over 5 min; and at 100% B for 5 min (the flow rate of all gradient steps was 0.5 mL/min). The major peak eluted at ca. 0.5 M NaCl (50% B) was precipitated with EtOH and dissolved in 10 mM Tris-HCl, pH 7.6, and 1 mM EDTA. Prior to formation of the RUMT–[^{3}H]methyl-FUtRNA complex, FUtRNA was heated at 80 °C for 5 min and then renatured by slowly cooling to room temperature.

Preparation and Purification of Enzyme–[^{3}H]Methyl-FUtRNA Complex. A reaction mixture (3.78 mL) containing RUMT (0.64 mg, 800 units/mg), FUtRNA^{Phe} (14.4 nmol), [^{3}H]methyl-AdoMet (200 nmol, 0.54 Ci/mmol), 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM DTT, and 50 mM NaCl was incubated at 18 °C. After 30 min, an additional 0.26 mg of RUMT in 0.8 mL of 20 mM Tris-HCl, pH 7.6, 0.5 mM DTT, 0.5 mM EDTA, and 20% w/v glycerol was added, and incubation was continued for 1.5 h at 18 °C.

The complex was purified by adsorption to a 1.5-mL DE-52 column (0.9 × 6 cm), equilibrated with 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 50 mM KCl. The column was washed with 10.2 mL of this buffer until tritium in the wash reached background (ca. 50 dpm/mL) and was then washed with 11.9 mL of 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 250 mM KCl to remove excess protein. The complex was eluted in 1.7 mL of 20 mM Tris-HCl, pH 76.1, 1 mM EDTA, and 1 M KCl and concentrated/desalted by precipitation with EtOH.

Lysylendopeptidase Digestion of RUMT–[^{3}H]Methyl-FUtRNA Complex. RUMT–[^{3}H]methyl-FUtRNA complex (1.8 nmol) in 100 μL of 100 mM Tris-HCl, pH 8.9, and 2 M Gdn-HCl was digested with 0.045 unit of LEP at 30 °C. After 70 min of incubation, EDTA and DTT were added to final concentrations of 2.5 and 20 mM, respectively, and an additional aliquot of LEP (0.045 unit) was added; the sample was flushed with argon and incubated for 60 min at 30 °C. The extent of digestion was assayed by monitoring the shift in electrophoretic mobility, using 7 M urea–15% PAGE and EtdBr to stain RNA. After complete proteolytic digestion, the peptide–[^{3}H]methyl-FUtRNA complex was precipitated with EtOH.

Separation of tRNA Peptides by 7 M Urea–15% PAGE and Electroblotting to PVDF Membrane. The EtOH-precipitated peptide–[^{3}H]methyl-FUtRNA complex was dissolved in water and then diluted with an equal volume of formamide containing 0.03% bromophenol blue and 0.03% xylene cyanole. The sample (ca. 400 pmol) was loaded to a 7 M urea–15%-polyacrylamide "minigel" (8 cm × 10 cm × 0.75 mm) as follows: Five percent was loaded to one lane of the gel to serve as a marker, and the remainder was loaded to an adjacent lane. Electrophoresis was carried out at 200 V until the xylene cyanole band reached the bottom of the gel. Following electrophoresis, the lane containing the marker was excised and stained with EtdBr. The remainder of the gel was electroblotted to Problott PVDF membrane in a Bio-Rad Mini-Transblot electrophoretic transfer cell. Electroblotting was carried out for 35 min at 50 V in CAPS/MeOH, pH 11, as described in Applied Biosystems' ProBlott brochure. To localize the peptide–[^{3}H]methyl-FUtRNA complex on the membrane, the PVDF strip was sliced horizontally into twelve 0.5 cm × 1.5 cm segments. One-tenth of each segment (0.5 cm × 0.15 cm) was suspended in 5 mL of Aquasol II and the radioactivity measured by liquid scintillation counting. The PVDF segment exhibiting the greatest amount of radioactivity and containing the putative peptide–tRNA was loaded to a gas-phase protein sequencer, and N-terminal sequencing was preformed.

[^{14}C]Carbamoylmethylation of Peptide–[^{3}H]Methyl-FUtRNA Complex. Following LEP digestion, the peptide–[^{3}H]methyl-FUtRNA complex was precipitated with EtOH to remove excess DTT. The pellet was washed with cold 70% EtOH, dried briefly under vacuum, and dissolved in 60 μL of 0.1 M Tris-HCl, pH 8.9, and 5.33 M Gdn-HCl containing 1 μCi of [^{14}C]iodoacetamide (59 mCi/mmol). The sample was flushed with argon and incubated for 60 min at 23 °C. The complex was precipitated EtOH, and the pellet was washed with cold 70% EtOH until the ^{14}C in the supernatant reached a constant value (ca. 200 dpm/mL).

V-8 Protease Digestion of the [^{14}C]Peptide–[^{3}H]Methyl-FUtRNA Complex. To 125 pmol of [^{14}C]peptide–[^{3}H]-methyl-FUtRNA in 100 μL of 0.1 M Tris-HCl, pH 7.5, was added 2 μg of V-8 protease. Following incubation at 37 °C for 2 h the complex was precipitated with EtOH, washed with cold 70% EtOH, and dissolved in water. Aliquots from both supernatant and dissolved pellet were counted in Aquasol II to quantitate ^{14}C and monitor the extent of proteolytic digestion. Since these results indicated incomplete digestion, the dissolved pellet was adjusted to 0.1 M Tris-HCl, pH 7.5, an additional 2 μg of V-8 protease was added, and the sample was incubated at 37 °C for 1 h. The peptide–[^{3}H]methyl-FUtRNA was recovered by EtOH precipitation and dissolved in 60 μL of water. Half of this solution was subjected to

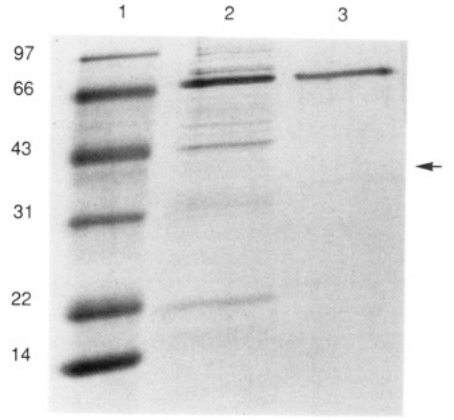

FIGURE 1: Purification of the covalent complex. Shown is a Coomassie-stained SDS gel of crude complex (lane 2) and the DEAE-purified complex (lane 3). Lane 1 shows the migration of standard molecular mass markers (97.4, 66.2, 42.7, 31.0, 21.5, and 14.4 kDa). The arrow marks the position of free RUMT.

gas-phase Edman degradation.

Covalent Protein Sequencing of the [^{14}C]Peptide–[^{3}H]-Methyl-FUtRNA Complex. The [^{14}C]peptide–[^{3}H]-methyl-FUtRNA (250 pmol) was attached to a DITC Sequalon membrane via the ϵ-amino group of the carboxy-terminal lysine, as described in the bulletin provided by the manufacturer of the membrane. Edman degradation (O3CPTH cycle) and PTH analyses were performed for nine cycles as described above, except that the O3CBGN cycle was omitted. After nine PTH cycles, solvent 1 (S1, *n*-heptane), solvent 2 (S2, ethyl acetate), and solvent 3 (S3, butyl chloride) were changed to ethyl acetate/*n*-heptane (50:50 v/v) (S1′), 100% MeOH (S2′), and 20 mM sodium phosphate, pH 7.0/MeOH (10:90 v/v) (S3′) (Wettenhall et al., 1990). ATZ cycles were then commenced, as described (Wettenhall et al., 1990), with minor modifications: to prevent blockage by salt precipitation, the ATZ transfer line was changed from 0.3 i.d. to 0.5 i.d., and after each cycle the line was extensively washed with S2′. Each ATZ fraction was counted in 4 mL of Aquasol II until the standard error was less than 1%.

RESULTS

Upon formation of the RUMT–[^{3}H]methyl-FUtRNA complex, the apparent molecular mass of RUMT shifted from 42 to 67 kDa on SDS–PAGE (Santi & Hardy, 1987). The 67-kDa band was approximately 50% pure as indicated by SDS–PAGE. Due to its RNA component, the complex eluted from DEAE-cellulose in high salt, whereas contaminating proteins eluted in medium salt. Using a RUMT preparation that was about 50% pure, DEAE-cellulose purification gave a complex that was greater than 95% homogeneous (Figure 1). The purity of the complex was also assessed by N-terminal protein sequencing, which yielded homogeneous PTH-amino acids for five cycles and confirmed the N-terminus of RUMT (data not shown).

The LEP digestion of the purified RUMT–[^{3}H]methyl-FUtRNA complex was monitored by 7 M urea–15% PAGE. After 70 min at 30 °C several FUtRNA–peptides were observed, which, upon continued incubation, converged to a single band, migrating slightly slower than free FUtRNA (data not shown). In a parallel experiment the gel was blotted to a PVDF membrane and the peptide–[^{3}H]methyl-FUtRNA complex was localized on the electroblot by measuring the radioactivity in segments of the membrane (Figure 2). The PVDF segment exhibiting the highest number of recovered counts corresponded to the putative peptide–[^{3}H]methyl-FUtRNA complex, as determined by EtdBr staining.

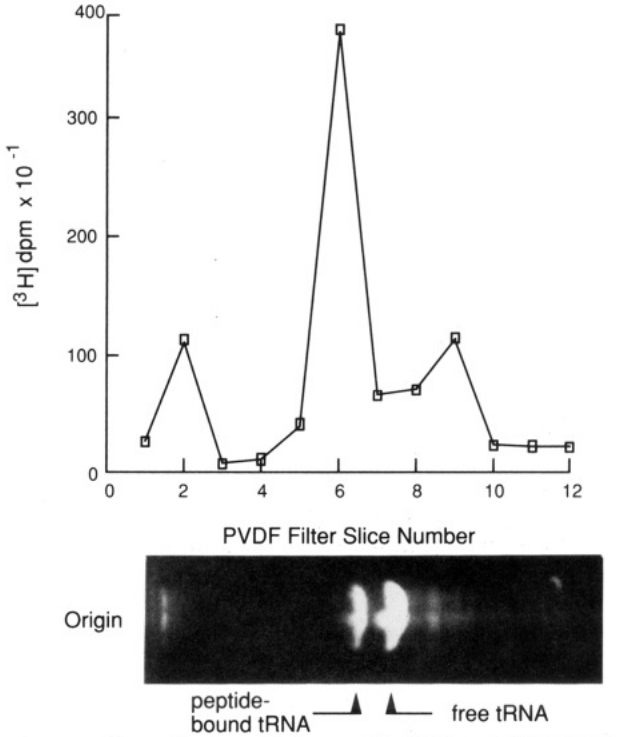

FIGURE 2: Electroblotting of the peptide–[^{3}H]methyl-FUtRNA complex to PVDF membrane. Shown is an ethidium bromide stained portion of a 7 M urea–15% polyacrylamide gel (depicted horizontally from the origin) contiguous to the gel section which was electroblotted to PVDF membrane. Disintegrations per minute (dpm) per PVDF slice is plotted, such that it corresponds to gel position.

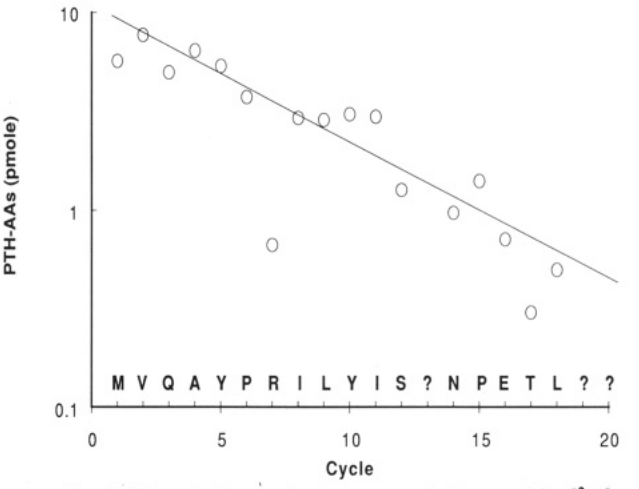

FIGURE 3: N-Terminal protein sequence of the peptide–[^{3}H]-methyl-FUtRNA complex. The theoretical initial PTH yield was 10.7 pmol, and average repetitive yield was 85%.

The PVDF segment containing the complex was subjected to 22 cycles of Edman degradation. With the exception of cycle 13, unambiguous amino acid assignments could be made for 18 consecutive cycles (Figure 3). When compared to the DNA sequence of RUMT (Gustafsson et al., 1991), the LEP fragment that remained attached to FUtRNA corresponded to residues 312–331: MVQAYPRILYISCNPETLCK. The C-terminus of the peptide was presumed to be Lys 331, since LEP cleaves at the C-terminal side of lysine. Thus, the catalytic nucleophile was concluded to be either Cys 324 or Cys 330 of the protein. However, a definitive assignment could not be made since neither Cys residue could be directly identified under normal operation of the gas-phase sequencer.

In order to distinguish free cysteine from nucleotide-bound cysteine, the peptide–[^{3}H]methyl-FUtRNA complex was

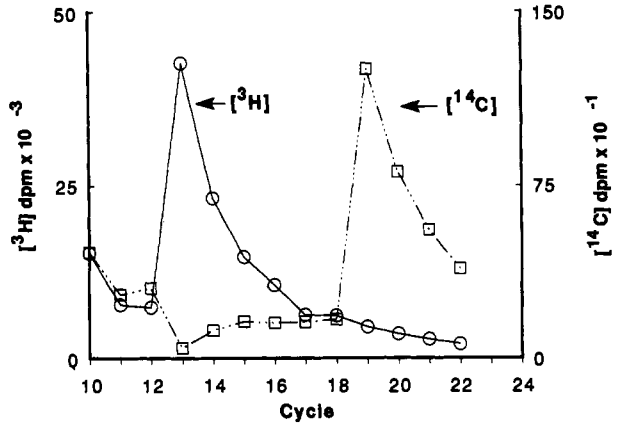

FIGURE 4: Covalent protein sequencing of the [^{14}C]peptide–[^{3}H]-methyl-FUtRNA complex. For Edman degradation cycles 10–22, ATZ amino acids were collected and quantitated by scintillation counting. The ATZ yields in cycles 13 and 19, as determined by radiochemical quantitation, were 36 and 10 pmol, respectively. The relative picomole yields of ^{3}H and ^{14}C are consistent with the theoretical initial yield of the peptide.

treated with [^{14}C]iodoacetamide to alkylate and label the free thiol. This yielded a doubly labeled complex in which one of the cysteines was labeled with ^{3}H and the other with ^{14}C. The [^{14}C]peptide–[^{3}H]methyl-FUtRNA complex was digested with V-8 protease, which cleaves at the C-terminal side of glutamic acid. Proteolysis should occur at Glu 327 and yield two peptides, one containing Cys–[^{3}H]methyl-FUtRNA, and the other containing [^{14}C](carbamoylmethyl)cysteine. The peptide that contained [^{3}H]FUtRNA was purified by EtOH precipitation and washed free of the [^{14}C]peptide (over 99% removal). The peptide that remained attached to [^{3}H]FUtRNA was sequenced and found to contain the same N-terminus as did the peptide prior to protease removal of the ^{14}C-modified cysteine. Further, the sequence abruptly terminated at Glu 327 (data not shown). This provides indirect evidence that cysteine 324 of the original LEP fragment is attached to FUtRNA and that cysteine 330 is carbamoylmethylated.

To definitively demonstrate that cysteine 324 is bound to FUtRNA, we sought to identify the sequencer cycles in which ^{3}H and ^{14}C were released and to correlate those cycles with the known peptide sequence. This approach is not feasible by "noncovalent" gas-phase sequencing due to the inability to extract [^{3}H]FUtRNA-Cys from a glass fiber or PVDF membrane, during conventional operation of the gas-phase protein sequencer. To recover ATZ–[^{3}H]FUtRNA–Cys, more rigorous extraction conditions were required, which necessitated covalent attachment of the [^{14}C]peptide–[^{3}H]methyl-FUtRNA to a solid support. The [^{14}C]peptide–[^{3}H]methyl-FUtRNA was covalently attached to a Sequalon DITC membrane through the ϵ-amino group of the putative C-terminal lysine, conventional protein sequencing was performed for nine cycles (PTH derivatives analyzed), and then ATZ-amino acids were collected by use of vigorous extraction conditions. As shown in Figure 4, tritium is released in cycle 13 and ^{14}C in cycle 19. These results clearly establish that Cys 324 is bound to FUtRNA and Cys 330 is carbamoylmethylated.

Discussion

The objective of this work was to identify the nucleophilic catalyst of RUMT which is believed to form a transient covalent adduct with U54 of tRNA. The strategy was to form a RUMT–[^{3}H]methyl-FUtRNA covalent complex, which mimicked a steady-state intermediate of the normal enzymic reaction, to convert the complex to a small peptide–[^{3}H]-methyl-FUtRNA complex, and then to sequence the peptide to determine which amino acid was attached to the RNA. The covalent complex was formed with RUMT, [^{3}H]methyl-AdoMet, and FUtRNA, purified from extraneous proteins by exploiting properties of RNA of anion-exchange chromatography, and treated with LEP to convert it to a peptide–[^{3}H]methyl-FUtRNA complex. The presence of tRNA in the complex allowed the peptide–RNA complex to be purified and desalted by simple EtOH precipitation, and to be visualized on PAGE by EtdBr staining.

The active site peptide had the 20 amino acid sequence MVQAYPRILYISCNPETLCK which contained two cysteine residues that were candidates for the nucleophilic catalyst. The cysteine covalently attached to [^{3}H]methyl-FUtRNA could not be identified by conventional protein sequencing because it was not possible to extract the modified amino acid from a glass fiber or PVDF membrane. To unequivocally identify the catalytic nucleophile, two complementary strategies were employed.

First, the peptide–[^{3}H]methyl-FUtRNA complex was reacted with [^{14}C]iodoacetamide to produce a peptide doubly labeled at the two cysteine residues. The [^{14}C]peptide–[^{3}H]methyl-FUtRNA was then cleaved with V-8 protease at the Glu between the two cysteines, and the peptide that remained attached to FUtRNA was isolated and sequenced. It was determined that the peptide containing Cys 324 remained attached to FUtRNA, and the peptide containing Cys 331 was released from the complex.

In a second approach, the [^{14}C]peptide–[^{3}H]methyl-FUtRNA was covalently attached to a PVDF membrane, and the peptide component was sequenced by using a modified Edman degradation protocol. After nine conventional sequencer cycles, ATZ-amino acids were collected by using rigorous extraction conditions; tritium was released at Cys 324 and ^{14}C at Cys 331.

These results unequivocally show that Cys 324 of RUMT is the residue attached to [^{3}H]methyl-FUtRNA in the covalent complex. We believe that the interaction of the mechanism-based inhibitor, FUtRNA, with RUMT mimics that of the natural tRNA substrate, so we conclude that Cys 324 is the nucleophilic catalyst in the normal enzyme reaction.

We can deduce that nucleophilic catalysis by RUMT requires substantial conformational change of the tRNA substrate. In the crystal structure of yeast tRNA^{Phe}, C6 of U54 is completely solvent inaccessible. Access to C6 is precluded from above and below the plane of the pyrimidine ring by base stacking of U54 between adjacent residues, Ψ55 and G53. Therefore, at an early stage of the reaction, the structure of the T-loop must be opened so that the sulfhydryl of Cys 324 can access C6 of U54 to form the covalent adduct. RUMT might accomplish this either by facilitating a conformational change in the RNA upon binding or by stabilizing a high-energy conformation of the RNA, where C6 of U54 is solvent accessible. It has recently been shown that the methyl-transfer step occurs by direct displacement of the C5 anion equivalent on the sulfonium ion of AdoMet (J. T. Kealey, S. Lee, H. G. Floss, and D. V. Santi, unpublished experiments). This places further constraint on the structure of the T-loop during methylation, since C5 and the methyl electrophile must be colinear for S_N2 displacement.

Several other proteins are believed to form Michael adducts with RNA and DNA as a means of enhancing binding specificity and catalysis. These include other RNA–pyrimidine methyltransferases, DNA–cytosine methyltransferases, pseudouridine synthases, and other unrecognized proteins (Santi et al., 1978; Santi & Hardy, 1987). The approach used here

for *Escherichia coli* RUMT should be applicable to the purification of other proteins which form stable Michael adducts, as well as identification of nucleophilic residues involved in covalent bond formation.

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Protein Disulfide Isomerase Appears Necessary To Maintain the Catalytically Active Structure of the Microsomal Triglyceride Transfer Protein†

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ABSTRACT: Protein disulfide isomerase (PDI) is a component of the microsomal triglyceride transfer protein (MTP) complex. This study was initiated to help elucidate the role of PDI in MTP. The 88-kDa polypeptide of MTP (88K) was dissociated from PDI by using chaotropic agents ($NaClO_4$ and KSCN), low concentrations of a denaturant (guanidine hydrochloride) or a nondenaturing detergent (octyl glucoside). As assessed by fluorescence and circular dichroism spectroscopy, these three different approaches appeared to dissociate the components of MTP under mild, nondenaturing conditions. The dissociating agents were diluted or removed by dialysis, and the free PDI and 88K were further characterized. In all cases, the dissociation coincided with the loss of triglyceride transfer activity. The free 88-kDa polypeptide readily aggregated, suggesting that it is a hydrophobic peptide. Even in the presence of chaotropic agents, when 88K was not aggregated, transfer activity was not expressed. These results suggest that the association of PDI with 88K is necessary to maintain the catalytically active form of the triglyceride transfer protein and prevent the aggregation of 88K.

The microsomal triglyceride transfer protein (MTP)[1] is found in the lumen of microsomes isolated from the liver or intestinal mucosa (Wetterau & Zilversmit, 1984, 1986). It facilitates the transfer of triglyceride (TG) between synthetic membranes or plasma lipoproteins (Wetterau & Zilversmit, 1985). MTP also catalyzes the transport of cholesteryl ester (CE) and to a lesser extent phosphatidylcholine (PC). The protein from bovine liver has been purified and characterized. It is a protein complex of molecular weight 150000, containing one 58000

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[1] Abbreviations: TG, triglyceride; CE, cholesteryl ester; PC, phosphatidylcholine; MTP, microsomal triglyceride transfer protein; 88K, subunit of the microsomal triglyceride transfer protein which has a molecular weight of 88000; PDI, protein disulfide isomerase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CD, circular dichroism.

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