Articles

Assembly of Protein−RNA Complexes Using Natural RNA and Mutant Forms of an RNA Cytosine Methyltransferase

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This work reveals that mutant forms of RNA methyltransferases that form 5-methylcytosine (m^5C) have characteristics that may make them useful for biomacromolecular assembly. The experiments utilized bacterially expressed Trm4p, a tRNA methyltransferase cloned from *Saccharomyces cerevisiae*. Like DNA m^5C methyltransferases, Trm4p mediates methylation using a covalent intermediate, which would allow Trm4p to be trapped as a stable protein−RNA complex when the substrate RNA contains a modified cytosine base such as 5-fluorocytosine. However, mutant forms of Trm4p are identified that fail to release RNA resulting in the formation of denaturant stable methyltransferase−RNA complexes that contain only natural nucleotides. The ability to form stable complexes with natural RNA gives these mutant forms of Trm4p greater potential versatility for biomacromolecule construction applications than the wild-type Trm4p enzyme or DNA methyltransferases for which the trapping of the covalent intermediate requires the presence of a nucleotide analogue at the site of modification.

Introduction

RNA m^5C methyltransferases are the most recently characterized family of proteins that transfer a methyl group to carbon 5 of a pyrimidine base.[1−3] They use a covalent intermediate formed by the thiol of an active site cysteine to aid the methyl transfer reaction.[4] This is a mechanism shared by all enzyme families that modify pyrimidines at carbon five, including the thymidylate synthases, DNA m^5C methyltransferases, RNA m^5U methyltransferases, and bacteriophage deoxycytidine hydroxymethyltransferases. However, one thing sets the RNA m^5C methyltransferases apart from the other enzyme families: the presence of a second conserved cysteine within the active site.

Direct evidence for covalent catalysis by any of these pyrimidine modifying enzymes can be obtained by trapping the enzyme as a denaturant resistant complex with a substrate molecule that contains a nucleotide analogue. The most commonly used analogues are 5-fluoro derivatives of uracil and cytosine or 5-aza derivatives of cytosine. Stable links formed in this way have also been utilized as an approach for the specific and irreversible targeting of DNA methyltransferases to sites within a synthetic DNA molecule. For this approach to work, the modified nucleotide must be present as the target base in the particular sequence context recognized by the DNA methyltransferase. Despite the requirement for DNA that contains modified nucleotides at specific sites, this methodology has been shown to provide a functional basis for DNA−protein biomacromolecular assembly.[5,6]

Recent studies have begun to demonstrate the possibility of RNA based nanoconstruction.[7,8] Therefore, proteins that can form stable covalent complexes with RNA molecules could be useful for the formation of protein−RNA structures. Nucleotide derivatives incorporated into RNA can trap covalent complexes formed by RNA m^5C and m^5U methyltransferases,[4,9] so the synthesis of RNA molecules containing base analogues could be used for protein−RNA complex assembly in an analogous approach to that described previously for DNA−protein construction. However, the unique active site arrangement of the RNA m^5C methyltransferases may provide a novel approach for protein−RNA complex assembly that would allow the use of RNA that contains only natural ribonucleotides.

RNA m^5C methyltransferases are cysteine rich proteins, but only two cysteines are fully conserved across the protein family.[3] Remarkably, both conserved cysteines are in sequences that resemble active site motifs involved in covalent catalysis.[4] One cysteine (PC−Cys) is in a ProCysSer sequence within a region that resembles the active site motif of DNA m^5C methyltransferases. The second conserved cysteine (TC−Cys) lies within a ThrCysSer sequence in a region that has limited similarity to the active site motif of the RNA m^5U methyltransferase TrmA, the enzyme that forms the riboT base in tRNA molecules. Although it was anticipated that the PC−Cys would be involved in covalent catalysis, it was discovered in experiments using the Fmu enzyme and a 5-fluorocytosine containing substrate that covalent catalysis is mediated by the TC−Cys.[4] An early study of the yeast Nop2 protein[10] had indicated a critical role for the PC−Cys, and further studies of the yeast Trm4p and Nop2p proteins showed that the PC−Cys is critical for release of methylated RNA.[11] Since then, structural studies of the Fmu enzyme and a putative RNA m^5C methyltransferase have shown that the pair of conserved cysteines are in close proximity within the active site.[12,13] The PC−Cys is now proposed to aid in the

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Table 1. Oligonucleotides Used for Site-Directed Mutagenesis

mutant	coding strand oligonucleotide sequence[a]	restriction site generated
C260S	CTGTGTGACGTTCCGAGCTCTGGTGATGGTACCATG	SacI
C310S	CAACGGTAGATTGGTTTACTCGACGTCTTCTTTAAATCCTATTGAAAATG	AatII

[a] Location of new restriction site generated by the mutation is underlined.

Table 2. Plasmids Used for Trm4p Expression

pET28b based bacterial expression plasmids				
plasmid	marker	mutation	His_6 protein encoded	ref
pRKNCL1	Kanamycin	none	Trm4p (Ncl1p)	*a*
pRK-PA	Kanamycin	C260A	PA-Trm4p	*a*
pRK-PS	Kanamycin	C260S	PS-Trm4p	this paper
pRK-TS	Kanamycin	C310S	TS-Trm4p	this paper
pRK-DM2	Kanamycin	C260S + C310S	PSTS-Trm4p	this paper

[a] See ref 11.

extraction of a proton from carbon 5 after methyl transfer, which is the first step in the β-elimination reaction required for the release of the modified RNA.[11,12]

In the work reported here, the active site cysteines of the tRNA methyltransferase Trm4p are replaced with serine, and the properties of the resulting proteins are characterized. In addition, we utilize two PC−Cys mutants of Trm4p (C260A and C260S) to form stable RNA−protein complexes in vitro. The complexes are formed due to faulty product release by the modified Trm4p enzyme and contain only natural ribonucleotides within the RNA.

Materials and Methods

Radiolabeled *S*-adenosyl-L-methionine [3H-methyl] was a product of MP Biochemicals (www.mpbio.com). The Talon affinity resin was purchased from Clontech. Stratagene was the source of BL21-Codon Plus (DE3)-RIL cells and the QuikChange mutagenesis kit. Protein molecular weight markers (broad range, prestained) were from BioRad as were the Quantum Prep Miniprep kits used for small scale plasmid preparations. Oligonucleotides were synthesized by Integrated DNA Technologies (www.idtdna.com), and those used for site-directed mutagenesis were purified by polyacrylamide gel electrophoresis prior to use. Whatman DE81 DEAE cellulose filters (2.5 cm diameter) were purchased through Fisher Scientific. General chemical reagents and buffers were products of Sigma-Aldrich Co.

Site-directed mutagenesis of the two active site cysteines in Trm4p was carried out by the previously described procedure using a pair of complementary oligonucleotides for each mutation.[11] The plus strand oligonucleotide for each mutation is shown in Table 1. Three mutant forms of Trm4p were constructed: PS-Trm4p contains the C260S mutation, TS-Trm4p corresponds to a C310S change, and PS•TS-Trm4p contains serine at both locations. DNA fragments containing each single and double mutation were inserted into a pET28 based Trm4p expression plasmid (pRKNCL1) using unique BglII and a HindIII sites as previously reported.[11] The presence of the desired mutations without additional changes was confirmed by sequencing the inserted DNA fragment in both directions for each construct using the NCL1SEQf (TGAACCATCTGGTTTCGTTGTAGC) and NCL1SEQr (TGTCATAGACAGGCCATTTGGA) primers. DNA sequence analysis was conducted by the Biochemistry Biotechnology Facility of the Indiana University School of Medicine in Indianapolis, IN using a Perkin-Elmer Applied Biosystems 377 XL DNA sequencer.

All forms of the Trm4p protein expressed in this work have six sequential histidines within a 20 amino acid−amino terminal extension that is encoded by the pET28 plasmid. The Trm4 gene is under the control of a T7 promoter in the expression plasmid, so the plasmids were transformed into the BL-21(DE3) strain of *Escherichia coli* for expression. Substantial production of Trm4p depends on the presence of additional tRNAs generated from the CodonPlus plasmid. The various forms of Trm4p were isolated from frozen cells and purified using Talon metal affinity resin as reported.[11] Protein concentrations were determined with the Coomassie Protein Assay reagent from Pierce using cytochrome *C* as the standard.

Enzyme assays were set up as reported,[14] but the determination of radiolabel incorporation utilized DEAE ion exchange filters to separate free ^{3}H-AdoMet from radiolabels incorporated into RNA. Samples of the assay (25 μL) were added to 1 mL of 50 mM glycine adjusted to pH 2.3. The diluted sample was applied to a 25 mm circle of DEAE cellulose using vacuum filtration. The filter was rinsed 5 times with 2 mL of 50 mM glycine pH 2.3 and twice with 2.5 mL of 70% ethanol and then counted in 5.5 mL of scintillation fluid.

Small RNA substrates were extracted from either a yeast *Trm4* deletion strain (YPW17) or a genetically similar Trm4p expressing strain (YPW16).[15] Total RNA was isolated and then fractionated by ion exchange essentially by the procedure of Knapp,[16] but the RNA was not ^{32}P-phosphate labeled for routine assays. Further details of the RNA preparation were previously reported.[14]

SDS−PAGE[1] of protein samples utilized 1.5 mm 8−18% polyacrylamide gels using previously reported buffers.[17] Gels were stained with Brilliant Blue R or prepared for autoradiography by fixing in 2-propanol/water/acetic acid (25:65:10) for 30 min followed by soaking in 75 mL of Amplify (Amersham) for 20 min prior to drying with a heated vacuum drier. Dried gels were exposed to preflashed Fugi Super RX film at −80 °C.

Results

Formation of PA-Trm4p-RNA Complexes. When PA-Trm4p (C260A mutation) is expressed in bacteria, nearly all of the isolated protein is linked to bacterial RNA.[11] The ability of PA-Trm4p to form RNA complexes in vitro was examined using a typical preparation in which only a small fraction of the PA-Trm4p was not already linked to RNA. To detect new complex formation in the presence of preexisting complexes, PA-Trm4p was incubated with ^{32}P-labeled RNA from the yeast Trm4 deletion strain. This RNA lacks 5-methylcytosine residues, and it formed radiolabeled complexes with PA-Trm4p when AdoMet was present, but ^{32}P-labeled complexes were not observed in the absence of AdoMet (Figure 1). The complexes probably contain tRNA based on the substrate specificity of Trm4p, but

(1) Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMet, S-adenosyl-L-methionine; m^{5}C, 5-methylcytosine; m^{5}U, 5-methyluridine; DEAE, diethylaminoethyl; DTT, dithiothreitol; MOPS, 3-(N-morpholino) propane sulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

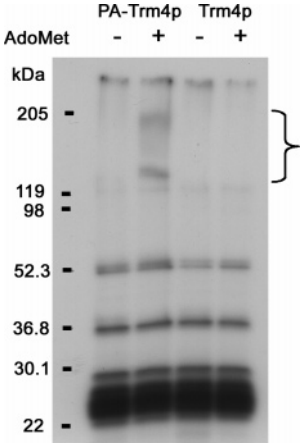

Figure 1. Formation of PA-Trm4p-RNA complexes. Small yeast RNAs were prepared from a Trm4 (Ncl1) knockout strain (YPW17) after labeling cells with ^{32}P-phosphate. Purified proteins (6 μg) were incubated with 0.15 μg of ^{32}P-RNA in buffer that contained 100 mM MOPS pH 7.8, 100 mM ammonium acetate, 5 mM magnesium acetate, and 4 mM DTT. Each sample was incubated with or without 2.6 mM *S*-adenosylmethionine for 90 min at 30 °C. SDS sample buffer (4×) was then added, and the proteins were resolved by SDS−PAGE. The dried gel was exposed to X-ray film for 48 h with an intensifying screen. A bracket indicates the size range where PA-Trm4p complexes are observed.

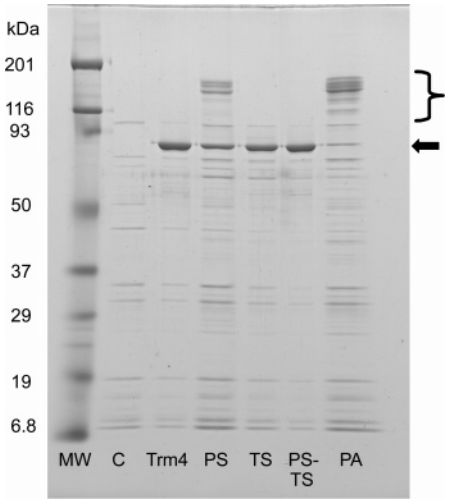

Figure 2. SDS−PAGE analysis of expressed Trm4p and mutant Trm4 proteins. Expressed proteins were purified using Talon affinity resin, resolved by SDS−PAGE, and the resulting gel was stained with Brilliant Blue R. Lanes contained prestained markers (MW), control extract from nonexpressing cells (C), 2 μg of wild-type Trm4p (Trm4), 4 μg of PS-Trm4p (PS), 2 μg of TS-Trm4p (TS), 2 μg of the PS·TS-Trm4p double mutant (PS-TS), and 4 μg of PA-Trm4p (PA). The position of tagged Trm4p is indicated by the arrow, and the size range for high molecular mass Trm4p-RNA complexes is indicated by the bracket. The control sample contained approximately 0.88 μg of protein in a volume equal to that of the largest Trm4p sample.

radiolabeled bands above the 30 kDa marker indicate the presence of some larger RNAs in the preparation: two in particular that comigrate with the 36 and 52 kDa protein markers.

The results indicate that RNA methylation is required for the formation of SDS−PAGE resistant complexes. Complex formation was not observed with wild-type Trm4p even when AdoMet and RNA were both present. Trm4p is catalytically active under these conditions, but the autoradiogram showed no evidence of the AdoMet dependent accumulation of denaturant resistant complexes with the wild-type enzyme. The lack of wild-type Trm4p complexes is made more significant by the fact that only a small fraction of PA-Trm4p is available for complex formation, while most of the wild-type Trm4p should be capable of interacting with RNA. Therefore, alteration of the PC−Cys (C260) is required for the formation of RNA-Trm4p complexes that are resistant to the denaturing conditions of SDS−PAGE.

Properties of PS-Trm4p, TS-Trm4p, and PS·TS-Trm4p. In an effort to further characterize the functional roles of the two cysteines found within the active site of RNA m^{5}C methyltransferases, the conserved cysteines were changed to serine by site-directed mutagenesis. After expression of each single and double replacement mutant and purification by metal affinity chromatography, a sample of each protein was analyzed by SDS−PAGE. The amino-terminally extended, but otherwise wild-type, Trm4p migrates with an apparent molecular mass of 85 kDa, which was also the size observed for TS-Trm4p and the PS·TS-Trm4p double mutant (Figure 2).

For PS-Trm4p, approximately half of the protein migrates at 85 kDa, but the remaining portion is resolved as a series of high-mass forms between 120 and 150 kDa. These large forms are within the same size range as those observed when PA-Trm4p is expressed in *E. coli*[11], and a sample of PA-Trm4p was included in the gel shown in Figure 2 for comparison. As previously shown for PA-Trm4p-RNA complexes,[11] the high mass forms of PS-Trm4p were shifted down to 85 kDa by treatment with RNase A, consistent with these larger forms of PS-Trm4p being protein−RNA complexes (data not shown).

PS-Trm4p is consistently produced as nearly equal amounts of the free 85 kDa enzyme and the larger RNA-linked forms, unlike PA-Trm4p, which is virtually all in RNA complexes. The substantially greater amount of free PS-Trm4p suggests that PS-Trm4p is either slower to form covalent complexes with RNA than PA-Trm4p or that PS-Trm4p releases RNA more rapidly. The latter would represent enzyme activity and seemed to be the most likely explanation given that serine chemically resembles cysteine much more than alanine. To determine the effect of the C260S mutation and the other serine replacements on enzymatic activity, all three mutant forms of Trm4p were assayed for methyltransferase activity using ^{3}H-methyl-labeled *S*-adenosylmethionine.

Enzyme assays were conducted as described in the Materials and Methods, and wild-type Trm4p shows considerable activity, but the serine replacement mutants have scant activity in comparison (Figure 3A). In Figure 3B, expansion of the vertical axis reveals that PS-Trm4p has an activity above that of the control extract, while also showing that the TS-Trm4p and PS·TS-Trm4p mutants are similar to the control extract. TS-Trm4p appears to possibly have a slight activity relative to the control in Figure 3, but additional assays using more enzyme and longer incubation times failed to demonstrate consistent activity over that of the control extract for various preparations of either TS-Trm4p or PS·TS-Trm4p double mutant (data not shown). The lack of activity for TS-Trm4p and PS·TS-Trm4p relative to control extracts will be discussed further when similar assay mixtures are analyzed by an alternative method.

At the 20 min time point in the assay shown in Figure 3, PS-Trm4p had incorporated 3.9% of the radioactivity transferred by the wild-type Trm4p. This seemed initially to indicate a 25-fold reduction in activity, but SDS samples prepared just prior to enzyme assays showed that approximately one-third of the PS-Trm4p remained in protein−RNA complexes 24 h after enzyme isolation (data not shown). Therefore, it is very difficult to accurately assess how active preparations of PS-Trm4p are relative to the wild-type enzyme. In addition, the stability of

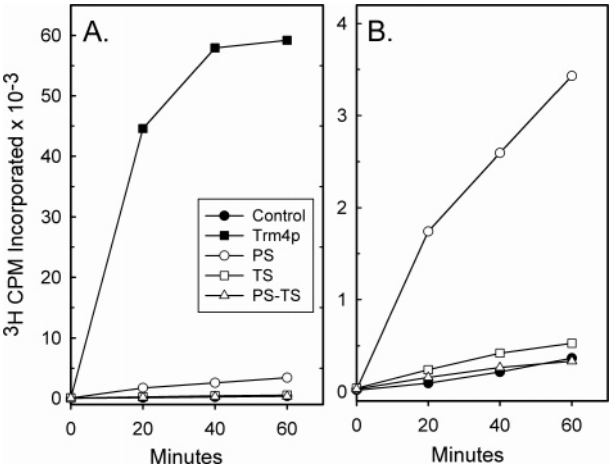

Figure 3. Assay of Trm4p methyltransferase activity. Trm4p activity was measured as described in the Materials and Methods using MOPS buffer adjusted to pH 7.8. Panel A compares the activity of wild-type Trm4p (filled squares) with PS-Trm4p (open circles), TS-Trm4p (open squares), PS-TS-Trm4p (triangles), and a control extract (filled circles). In panel B, the vertical axis is expanded and Trm4p data are deleted, to show the apparent activity of PS-Trm4p relative to the other mutants and the control. Plotted values are averages of duplicate assays.

the complexes formed with bacterial RNA during expression led to the concern that PS-Trm4p may form stable complexes with yeast RNA in the assay rather than producing free methylated tRNA. The DEAE ion exchange membranes used to separate free ^{3}H-AdoMet from radiolabeled tRNA in the assay would be expected to bind any radiolabel incorporated into enzyme−RNA complexes, and this would appear to be activity, although it would represent only the first half of the catalytic process.

To determine if protein−RNA adducts rather than free methylated tRNA are being produced by PS-Trm4p in the enzyme assay, reaction mixtures from a typical assay were stopped with SDS sample buffer and applied to SDS−PAGE gels. The polyacrylamide gels are able to resolve free enzyme from Trm4p-RNA complexes and methylated tRNA. Because of the substantially greater activity of wild-type enzyme, only one-tenth of the Trm4p samples was loaded onto the gel. The dried gel was exposed to film for 100 h, and the resulting autoradiogram is shown in Figure 4A. It revealed a series of five radiolabeled bands produced by the wild-type enzyme that correspond to the migration of tRNA molecules in the SDS−PAGE system (apparent size range of 25−35 kDa). In contrast, virtually all of the radiolabel incorporated by PS-Trm4p was in a band that had an apparent molecular mass greater than 116 kDa. This is significantly larger than the free enzyme and is within the size range observed for Trm4p-RNA complexes formed by the PA and PS-Trm4p mutants. A minor amount of radioactivity was observed in tRNA sized bands in the PS-Trm4p reactions, but the intensity was much less than that seen with the 10-fold diluted wild-type Trm4p. The actual amount of methylated tRNA produced by PS-Trm4p might be even less than that observed on the gel since some of the methylated tRNA may have been released from the enzyme after the addition of the SDS sample buffer rather than during the assay.

A longer exposure shown in Figure 4B confirms that the pattern of methylated RNA obtained with PS-Trm4p matches that observed for the wild-type Trm4p, except for the lighter intensity. The long exposure also allowed for the detection of minor methylated products in the TS-Trm4p and PS•TS-Trm4p reactions. However, these products do not appear to be the result of Trm4p activity because identical bands were generated by the control extract made from nonexpressing cells. In addition, the banding pattern of modified molecules differs somewhat from that observed with wild-type and PS-Trm4p. It seems most likely that a small amount of methylation occurs due to a residual *E. coli* enzyme and that the TS and PS•TS forms of Trm4p lack any significant activity.

Requirements for PS-Trm4-RNA Complex Formation. As seen in Figure 2, substantial amounts of free PS-Trm4p are formed when it is expressed in *E. coli*. When PS-Trm4p is eluted from the metal affinity resin, early fractions are enriched in complexes, but the later fractions are primarily free PS-Trm4p. By using material from late fractions, we were able to examine the requirements for complex formation using a gel-shift assay. In this method, the protein is shifted due to complex formation with RNA, and the interaction needs to be covalent due to the denaturing effect of SDS. The shift of PS-Trm4p from free protein to complexes requires the addition of both RNA and AdoMet, as no shift occurred when either was added alone (Figure 5, lanes 1−9). We also examined the ability of small RNAs from a Trm4p expressing yeast strain to form complexes.

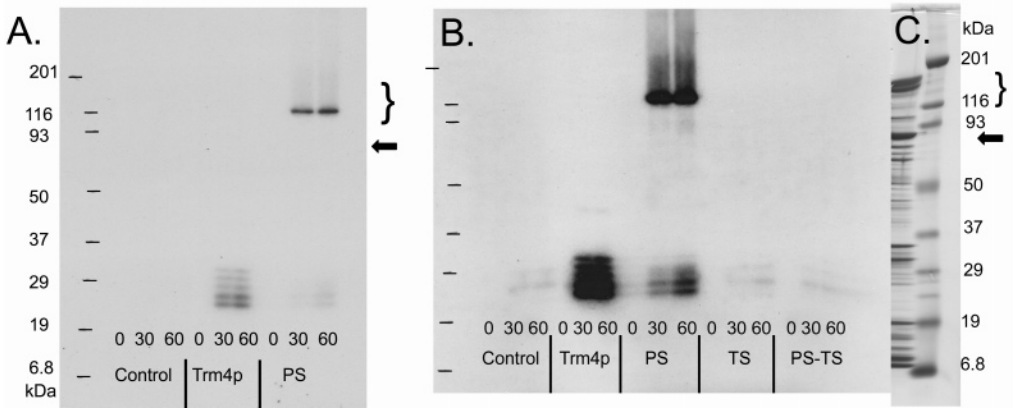

Figure 4. SDS−PAGE analysis of products from ^{3}H-AdoMet assay. Methyltransferase assays were set up using ^{3}H-methyl-labeled AdoMet and 0.5 μg of enzyme as done for the assays in Figure 3, but at selected times (0, 30, and 60 min) 35 μL of the 120 μL assay solution was mixed with 12 μL of 4× SDS sample buffer. Then, 40 μL of each sample was resolved by SDS−PAGE. For the wild-type enzyme, samples were diluted 10-fold prior to loading the 40 μL sample. Autoradiograms obtained from 100 h (A) and 1072 h exposures (B) are shown. Panel C shows the final two lanes of the gel that were removed, stained with Brilliant Blue R, and dried separately from the rest of the gel. The stained lanes contain two samples: PS-Trm4p in the left lane to show the size of the free enzyme (arrow) and the size range observed for Trm4p-RNA complexes (bracket) and prestained molecular mass markers in the right lane.

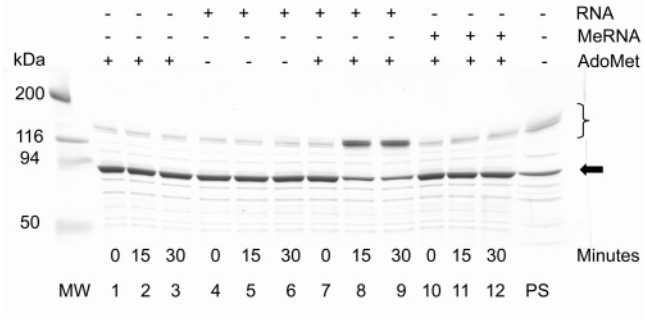

Figure 5. Formation of PS-Trm4p-RNA complexes. Four μg of PS-Trm4p was incubated with RNA and AdoMet as indicated for individual samples. When added, 3 μg of RNA was used, and the AdoMet concentration was 0.5 mM. Small yeast RNA molecules without 5-methylcytosine (RNA) were isolated from a Trm4 deletion strain. Methylated RNA (MeRNA) was isolated from Trm4 expressing yeast. At the times indicated, the reactions were terminated by the addition of 33 μL of 4× SDS sample buffer to each 100 μL reaction. To resolve free PS-Trm4p from PS-Trm4p-RNA complexes, 100 μL of each sample (~3 μg of PS-Trm4p) was resolved by SDS−PAGE, and the resulting gel was stained with Brilliant Blue R.

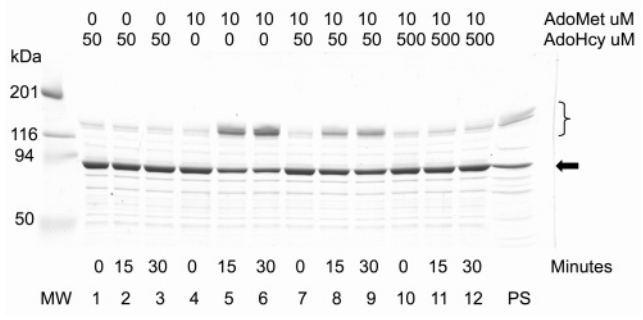

Figure 6. AdoHcy inhibits complex formation and cannot replace AdoMet. All reactions contained 4 μg of PS-Trm4p and 3 μg of small yeast RNA from the Trm4p deletion strain. AdoMet and AdoHcy were added as indicated for individual samples. After incubation at 30 °C for the indicated times, the reactions were terminated by the addition of 33 μL of 4× SDS sample buffer to each 100 μL reaction. Free PS-Trm4p was resolved from PS-Trm4p-RNA complexes by SDS−PAGE, and the resulting gel was stained with Brilliant Blue R. In each case, 3 μg of PS-Trm4p was loaded onto the gel.

This RNA (MeRNA in Figure 5) would be expected to be largely methylated at target cytosines, and lanes 10−12 of Figure 5 show that very little shift occurs with the methylated RNA. This result is consistent with the poor ability of small RNAs from Trm4p expressing yeast to serve as substrates in the methyl transfer assay with wild-type Trm4p enzyme. The RNA isolated from a Trm4p expressing strain accepts only one-tenth to one-seventh of the radiolabel incorporated into an equal amount of unmethylated RNA from a Trm4p deletion strain.

The initial formation of a Michael adduct between a methyltransferase and a pyrimidine base is proposed to form a readily reversible carbanion intermediate.[18] To be isolated, the carbanion needs to be stabilized by methylation or protonation.[18] It would be anticipated that AdoHcy would be able to cause the formation of SDS-stable complexes if the covalent intermediate forms and the target cytosine are protonated in the presence of AdoHcy. However, if methylation is required to stabilize the Michael adduct, then AdoHcy alone would not be able to cause complex stabilization due to its inability to donate a methyl group. A test of the ability of AdoHcy to drive complex formation between unmethylated RNA and PS-Trm4p in shown in Figure 6. At a concentration of 50 μM, AdoHcy fails to cause complex formation (lanes 1−3), while just 10 μM AdoMet results in the substantial upshift of PS-Trm4p (Figure 6, lanes 4−6). Although AdoHcy is unable to cause complex formation, it would be expected to inhibit AdoMet dependent complex formation. Addition of a 5-fold excess of AdoHcy over AdoMet significantly reduced complex formation (Figure 6, lanes 7−9), while a 50-fold excess of AdoHcy resulted in nearly total inhibition of complex formation (Figure 6, lanes 10−12).

Discussion

By using radiolabeled RNA, it was possible to show that PA-Trm4p can form denaturant resistant complexes in vitro. Because of the reversibility of Michael adducts, it was anticipated that the formation of SDS resistant complexes may depend on RNA methylation. The requirement for AdoMet is consistent with that hypothesis, but methyl group transfer was not directly demonstrated in the PA-Trm4p experiment shown in Figure 1. The modification of PC−Cys (C260 in Trm4p) is required for the observation of denaturant resistant complexes as none were observed with wild-type Trm4p.

Neither the methyl group transfer assay nor direct analysis of reactions by SDS−PAGE and autoradiography were able to demonstrate clear activity for the TS-Trm4p or the PS·TS-Trm4p mutants. The lack of detectable activity by these forms of Trm4p is consistent with the proposed role of TC−Cys in forming the Michael adduct, which is critical for catalysis. A serine replacement mutant of the active site cysteine in the EcoRII DNA m^5C methyltransferase has been reported to have residual activity; however, the activity of the mutant protein was reduced approximately 10 000-fold.[19] For the assays used here, any reduction in activity over 100-fold would probably be indistinguishable from the background. Therefore, we cannot say that the TS-Trm4p and PS·TS-Trm4p mutants are absolutely inactive, but they are certainly much reduced in activity.

The PS-Trm4p initially appeared to have low but significant activity in the methyl transfer assay, until the SDS−PAGE analysis revealed that the main product was RNA−protein complexes. The incorporation of radiolabels into denaturant resistant complexes confirmed that PC−Cys is not required for the formation of the covalent intermediate or for methyl group transfer. The slight production of free methylated tRNA indicates some low level of activity for PS-Trm4p. However, SDS−PAGE could cause an overestimation of activity since the RNA−protein complexes formed by PS and PA-Trm4p gradually disassemble in the SDS sample buffer. The high concentration of mercaptoethanol in the SDS sample buffer may be responsible for complex breakdown, nevertheless complex breakdown in the SDS sample buffer is slow at room temperature and even at 65 °C. The rate of disassembly increases when SDS samples of complexes are placed in a boiling water bath, resulting in an estimated half-life of 3−4 min (data not shown). To minimize complex breakdown, gel samples for the experiments shown were not heated.

The accumulation of PS-Trm4p-RNA complexes is consistent with the primary failure of this mutant being in the release of modified RNA. This is consistent with, but not proof for, the hypothesis that PC−Cys aids methyltransferase activity by acting as a base to extract a proton from carbon 5 following methylation, which initiates β-elimination and allows reversal of the Michael adduct that was formed by TC−Cys.[11,12]

Using fractions enriched with free PS-Trm4p, the gel-shift assay shown in Figure 5 confirmed that unmethylated RNA and

AdoMet are required for complex formation. Methylated RNA from a yeast strain that expresses Trm4p largely fails to cause complex formation. The minor amount of complexes formed may be the result of some unmethylated Trm4p sites in the isolated RNA or may result from sites not typically modified by Trm4p being available in the protein free RNA added to the reactions. The low level of complex formation is consistent with the reduced ability of methylated RNA to serve as an acceptor of ^{3}H-methyl groups in the methyl transfer assay.

AdoHcy is unable to cause complex accumulation as seen in Figure 6, but it can inhibit complex formation with unmethylated RNA. To have a strong inhibitory effect on complex formation, AdoHcy has to be present in an excess of AdoMet. All types of AdoMet dependent methyltransferases are inhibited by AdoHcy, the universal product of these methyl transfer reactions. The results shown in Figure 6 indicate that the RNA m^{5}C methyltransferases are subject to product inhibition like other AdoMet dependent methyltransferases.

For most enzymes that use a covalent intermediate during the modification of a pyrimidine base, denaturant resistant complexes have only been observed when the enzyme interacts with a nucleotide analogue such as those discussed in the Introduction, but in two cases, denaturant resistant complexes with normal nucleotides have been observed. The m^{5}U methyltransferase TrmA is able to form denaturant resistant complexes with natural RNA in the absence of AdoMet.[20] In addition, the putative DNA methyltransferase DNMT2 was found to form denaturant resistant complexes with synthetic DNA that contained only natural nucleotides.[21] It should be noted that recent studies have shown tRNA rather than DNA to be the natural substrate for DNMT2.[22] When denaturant resistant complexes are formed in the absence of a cofactor, stabilization of the Michael adduct probably results from protonation of the intermediate.[18] Several lines of evidence indicate that methylation and not just protonation is required to obtain stable complexes with PS-Trm4p. Unmethylated RNA fails to form complexes in the absence of AdoMet. Methylated RNA cannot replace unmethylated RNA nor can AdoHcy replace AdoMet. Furthermore, the presence of radiolabeled methyl groups in the PS-Trm4p-RNA complexes shown in Figure 4 supports the stabilization of complexes by methyl group addition. However, protonation could be cofactor dependent if the active site is not in the correct conformation to form a covalent Michael adduct until the cofactor is bound. The latter seems possible for RNA methyltransferases based on the structure of the Fmu enzyme, which shows the cofactor tucked into a pocket on one side of what appears to be an active site cleft.[12] Therefore, the AdoMet dependence of complex formation could be related to RNA methylation or cofactor dependent RNA binding. It may be possible to differentiate between these possibilities by examining the ability of an AdoMet analogue like sinfungin to cause complex formation. Sinfungin cannot donate a methyl group, but it might allow adduct formation if only protonation is needed to stabilize the covalent complex.

Although TrmA and DNMT2 can form denaturant resistant complexes, Trm4p is the first example of stable complex accumulation resulting from the mutation of a specific residue in the methyltransferase. The growth in the area of RNA nanotechnology suggests that there may be applications for RNA m^{5}C methyltransferases in RNA−protein macromolecular assembly. In some cases, protein−RNA complexes may be generated by replacing cytosine with 5-fluorocytosine or similar base analogues as has been done with DNA methyltransferases,[5,6] but the mutant forms of Trm4p reported here form denaturant resistant complexes with natural nucleotides. The advantage of the mutant enzyme is that the RNA does not need to contain modified nucleotides, and so RNA transcribed in vivo or in vitro could be used. It has already been shown that Trm4p will function with substrates that are smaller than a complete tRNA;[3] therefore, substrates will not have to be intact tRNA molecules. In addition, the protein products for most genes that encode putative RNA m^{5}C methyltransferases have not been characterized. Some particularly useful enzymes could be those expressed by thermophilic organisms. The apparent stability of complexes formed by the PA-Trm4p mutant may make it the best protein for complex assembly, but to be useful, it will be necessary to generate much more of the PA-Trm4p mutant in the uncomplexed form. Even though the PS-Trm4p-RNA complexes may gradually breakdown, the PS-Trm4p mutant could prove useful in developing the nanotechnology applications of these proteins. In addition to basic protein−RNA macromolecular construction, the PS-Trm4p could be useful in the detection of unmethylated tRNAs such as those from bacteria.

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References and Notes

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