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A single tRNA (guanine)-methyltransferase from *Tetrahymena* with both mono- and di-methylating activity

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ABSTRACT

A tRNA (guanine-2) methyltransferase has been purified to homogeneity from the protozoan Tetrahymena pyriformis. The enzyme methylates purified E. coli tRNAs which have a guanine residue at position 26 from the 5' end; it also methylates tRNA prepared from the $m^2_2G^-$ yeast mutant trm 1. This methyltransferase is therefore equivalent to the guanine methyltransferase 2mGII found in mammalian extracts. The purified 2mGII from Tetrahymena is capable of forming both N^2-methylguanine and N^2_2-dimethylguanine on a single tRNA isoaccepting species; under conditions of limiting tRNA or long reaction times the predominant product is dimethylguanine. Analysis of the products formed under varying reaction conditions suggests that dimethylguanine formation is a two step process requiring dissociation of the enzyme-monomethylated tRNA intermediate.

INTRODUCTION

Primary transcripts of tRNA molecules undergo diverse modification reactions during their maturation. An important group of modifications is the methylation of specific nucleoside residues. While the biological roles of these methylations are not clearly defined, changes in methylation patterns can alter the activity of tRNAs in in vitro reactions. Methylation of the guanine at position 10 in $tRNA^{phe}$, for example, influences the molecule's conformation (1) and alters its ability to be aminoacylated in vitro (2). Other methyl modifications have also been reported to affect the activity of tRNA molecules in in vitro protein synthesis reactions (3-5).

Several of the enzymes responsible for the methylation of mammalian tRNA have been isolated (6-12). One of these is the N^2-guanine methyltransferase, 2mGII, which has been partially purified from both rat liver and a mammary adenocarcinoma (11).

2mGII is defined by its ability to methylate guanine residues at position 26 to either N^2-mono- or dimethylguanine, in contrast to 2mGI which forms N^2-monomethylguanine at position 10 (12). An unusual property of 2mGII derived from adenocarcinomas was its ability to carry out low level methylation of tRNA molecules not containing G_{26} residues (11). However, detailed characterization of 2mGII was impeded by the difficulty of obtaining adequate amounts of the purified enzyme from mammalian tissues.

We now report the isolation, purification and properties of a 2mGII from Tetrahymena pyriformis. While the Tetrahymena enzyme differs in some respects from its mammalian counterparts, it has some properties similar to 2mGII from rat mammary adenocarcinoma.

MATERIALS AND METHODS

Enzyme assays.

Methyltransferase activity was determined using 125µl incubation mixtures as described previously (7). One unit of enzyme is defined as 1 nmol of methyl incorporated per 60 min. Assay of less purified enzyme (through DEAE cellulose) was in the presence of 40mM putrescine; more highly purified preparations were assayed with 20mM putrescine or 5-10mM magnesium acetate. E. coli MRE tRNA (Boehringer Mannheim) at a concentration of 20µM was used for routine methyltransferase assays. Purified tRNAs were obtained from the following sources: E. coli tRNAs from Subriden,Inc., Rolling Bay, WA.; yeast $tRNA^{Phe}$ from Boehringer Mannheim; tRNA from the yeast $m^2{}_2G^-$ mutant trm 1 was kindly provided by Dr. Anita Hopper (Hershey Medical Center, Hershey, PA.)

Identification of the products methylated by enzyme preparations was accomplished by HPLC separation of nucleosides from hydrolyzed tRNA samples (11).

Cell growth and harvest.

Cultures of Tetrahymena pyriformis W were maintained at 28° on a slant in capped tubes containing 4 ml pepticase medium [2% pepticase w/v, 0.1% yeast extract (Difco) and 90 µM Fe-EDTA]. Cells were passaged every 48 hrs (mid-stationary phase) by loop

inoculation. Seed cultures (100 ml medium in 500 ml Erlenmeyer flasks) were inoculated with one 48h stock culture and grown 20-24h (early stationary phase) at 28°C. Growth flasks (500 ml medium in 2800 ml Fernbach flasks) were inoculated with 4ml seed culture and incubated without shaking at 28° for 44-48h (mid-stationary phase).

Cells were harvested and washed with 200ml 10mM Tris-HCl buffer, pH7.5, using a modified continuous flow plankton centrifuge (13). Concentrated cells were pooled in 100ml oil centrifuge tubes and centrifuged 10 min at 300xg in an IEC HN-SII centrifuge. Excess buffer was aspirated and the cell pellet resuspended in an equal volume of DEAE buffer A (20% ethylene glycol; 50mM Tris-HCl buffer, pH 8.15; 80mM NaCl; 0.5mM EDTA and 10mM 2-mercaptoethanol).

The cell suspension was disrupted with ultrasound using a Wave Energy Systems dismembranator (Newton, Pa.), using 3 pulses of 15 seconds each, at 70 watts with the tubes kept in an ice bath. A high speed supernatant was prepared immediately by centrifugation at 135,000xg using a Beckman SW-28 rotor.

Enzyme purification

DEAE-cellulose chromatography. High-speed supernatant was applied to a 2.5x35 cm column of Whatman DE-52, pre-equilibrated with DEAE buffer A, at 2 ml/min. Fractions of 5.0ml were collected. The column was washed with DEAE buffer A with the NaCl concentration increased to 100mM immediately after sample application was complete. Elution with this buffer was continued until the absorbance descended steeply; 200 ml buffer containing 250mM NaCl was then applied. Following elution of an additional A_{280} absorbing peak, the column was washed with buffer containing 500mM NaCl and finally re-equilibrated with buffer A. DEAE columns were reused 3-4 times before decreasing recovery of enzyme activity necessitated discarding the column. Fractions were assayed and peaks containing tRNA methyltransferase activity were pooled and stored at -70°. The DEAE peak containing guanine-2 methyltransferase activity [peak A; Fig.1(a)] was used as starting material for further purification.

Phosphocellulose chromatography. Peak A from two DEAE columns

were combined and dialyzed 2X against 2L of phosphocellulose buffer (80mM potassium phosphate, pH6.5; 20% ethylene glycol; 0.5mM EDTA and 10mM 2-mercaptoethanol). The dialysate was applied to a 1.5x30cm column of phosphocellulose (Schleicher and Schuell) at 0.5ml/min. Unabsorbed material was washed from the column in starting buffer until a decrease in A_{280} was observed. A 300ml linear gradient (80mM-300mM potassium phosphate) was employed to resolve 2 peaks of methyltransferase activity (Fig 1b). The peak fractions were pooled and the volume of each reduced by diafiltration using Amicon PM-30 membranes. Enzyme was then divided into aliquots for storage at -70°.

Affinity chromatography. Homogeneous preparations of 2mGII were obtained by affinity elution (14) using a 1.5x4cm column containing matrex gel Blue A (Amicon) equlibrated with affinity starting buffer (10mM potassium phosphate, pH6.5, 10mM mercaptoethanol and 20% ethylene glycol). Approximately 150 units of phosphocellulose-purified enzyme (Peak A_I) were dialyzed 2h vs. the starting buffer and applied to the affinity gel at 15ml/h. After washing with 15ml starting buffer, bound tRNA methyltransferase activity was eluted with 12ml starting buffer containing 1.6µM yeast tRNA (Boehringer Mannheim). The column was washed with 10ml additional buffer without tRNA before the additional methyltransferase activity (and contaminating proteins) were eluted with buffer containing 300mM potassium phosphate.

Yeast tRNA was removed from the affinity-purified enzyme by chromatography on DE-52 using 50mM Tris-HCl, pH 8.15, 10mM 2-mercaptoethanol, 20% ethylene glycol with 80mM NaCl. The enzyme was then dialyzed against 10mM Tris, pH8.15, 10mM 2-mercaptoethanol and 40% glycerol. Dithiothreitol was added to a concencentration of 5mM, the enzyme was aliquoted and stored at -70°.

Gel filtration. The molecular mass of 2mGII was determined with Sephacryl-200 or -300 (Pharmacia); this procedure also served to remove residual ribonuclease activity from phosphocellulose purified enzyme. Two ml of the phosphocellulose enzyme were applied to a 1.5x52cm column equilibrated with 50mM Tris-HCl buffer, pH 8.15; 100mM NaCl, 10mM 2-mercaptoethanol and 20%

ethylene glycol. Elution rate was 6ml/hr and 0.5ml fractions were collected. Fractions containing activity were pooled, dialyzed vs. 10mM Tris-HCl pH 8.15, 10mM 2-mercaptoethanol, 40% glycerol and aliquots stored at -70°. Molecular weights were determined using the Sigma modifications of the methods of Whitaker (15) and Andrews (16) by comparison with elution profiles of authentic molecular weight standards (Sigma Chemical).

Polyacrylamide gel electrophoresis

Enzyme preparations were analyzed by electrophoresis under non-denaturing conditions on a variety of gel systems with polyacrylamide concentrations ranging from 4.5-10%. Molecular weight standards (Sigma Chemical Co. St. Louis) were run with all gels; proteins were visualized by staining with Coomassie Brilliant Blue R. The molecular mass was calculated using Ferguson plots (17) obtained by the Sigma modified techniques of Bryan (18) and Davis (19). Location of 2mGII activity after electrophoresis was verified by allowing the enzyme to diffuse out of 0.5cm gel sections into 50mM Tris-HCl, pH 8.15 containing 100mM NaCl, 10mM 2-mercaptoethanol and 20% ethylene glycol, for 2h at 4°. Gel eluates were then assayed for methyltransferase activity.

Protein measurement

Protein content of high speed supernatants and DEAE preparations was quantified using the Bio-Rad protein reagent. The protein content of highly purified preparations was determined by the method of Schaffner and Weissman (20).

RESULTS

Purification

Previous tRNA methyltransferase purification protocols reported from this laboratory (7,8,11) have employed ammonium sulfate fractionation followed by DEAE-Sephadex chromatography for initial steps in purification. Because of the high levels of proteases and nucleases in Tetrahymena, we chose to apply a high speed supernatant directly to DEAE cellulose, thus achieving rapid enzyme extraction and initial fractionation. The elution profile of Tetrahymena tRNA methylating activities

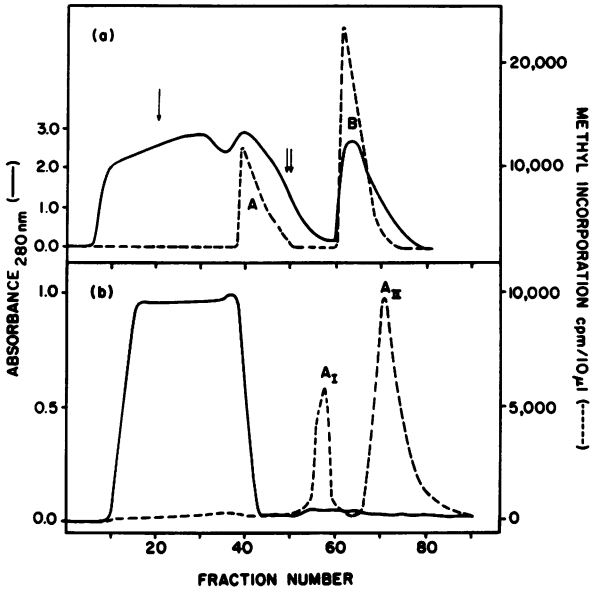

Fig.1. (a). DEAE-cellulose chromatography of Tetrahymena high speed supernate. The column was equilibrated with DEAE buffer containing 80mM NaCl. Peak A was eluted by increasing the NaCl concentration to 100mM (single arrow); a second peak of activity, Peak B was eluted after addition of buffer containing 250mM NaCl (double arrows). Fraction size was 5ml.
(b). Phosphocellulose chromatography of DEAE-cellulose Peak A. Two pooled DEAE peaks A were applied to a phosphocellulose column equilibrated in buffer containing 80mM potassium phosphate. Fraction size was 6ml; a 300ml linear gradient (80-500mM potassium phosphate) was started at fraction 40.

from DEAE cellulose is shown in Fig. 1a. Two peaks of activity are resolved; the first (Peak A) contains N^2-methylguanine (m^2G) and 1-methyladenine (m^1A) tRNA methyltransferase activity and the second (Peak B) contains 5-methylcytosine, 1-methyladenine and 1-methylguanine methyltransferase activities (Table I). Each of these peaks when pooled and stored at -70° were stable for at least 1 year.

Phosphocellulose chromatography of DEAE peak A (Fig. 1b) separated the guanine methyltransferase activity (Peak A_I) from the adenine methyltransferase (Peak A_{II}). Both peaks of enzyme activity were retained by Amicon PM-30 membranes; these membranes were used therefore to concentrate the enzymes before

Table 1. Distribution of tRNA methyltransferases during enzyme purification

	Methylated nucleoside formed				
	m^1Ado	m^5Cyd	m^1Guo	N^2-mGuo	$N^2{}_2$-mGuo
Enzyme preparation					
High speed supernate	39%	31%	2%	21%	7%
DEAE Peak A	64%	<1%	-	27%	8%
DEAE Peak B	24%	71%	5%	-	-
Phosphocellulose A_I	1%	-	-	73%	26%
Phosphocellulose A_{II}	100%	-	-	-	-

Enzyme aliquots were used to methylate E. coli K-12 tRNA. The methylated tRNA was hydrolyzed and the resulting nucleosides separated by HPLC. Results are expressed as percent of total counts in methylated nucleosides.

dialysis and storage. The N^2-guanine methyltransferase preparation derived from phosphocellulose is referred to as A_I. The A_I enzyme preparation frequently contained traces of RNase activity; when this preparation was used to examine properties of the enzyme (see below) the residual RNase was removed by gel filtration on Sephacryl 300.

A final step in 2mGII preparation was achieved by affinity elution from Amicon gel matrex Blue A using yeast tRNA(Fig 2). Approximately 20% of the methyltransferase activity was eluted from the column upon addition of tRNA; this preparation is referred to as "affinity A_I" enzyme. Additional methylating activity could be recovered when the salt concentration was increased to 300mM, however, the salt eluted enzyme was not homogeneous as judged by gel electrophoresis. The yield and extent of purification of the enzyme preparations is detailed in Table 2.

Chromatography of the A_I enzyme on Sephacryl 300 columns indicated a molecular weight of approximately 200kD. When the more highly purified affinity-A_I enzyme was electrophoresed under nondenaturing conditions and stained, a single band was observed at all polyacrylamide concentrations used (Fig 3). A molecular weight of 240-260kD was calculated for the stained band. The correspondence between the stained band and tRNA

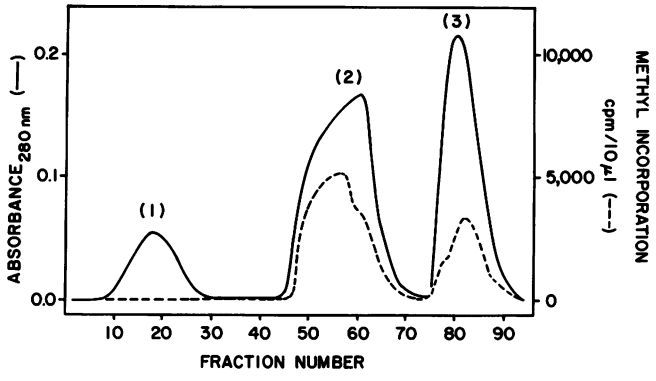

Fig.2.Matrex gel blue A chromatography of phosphocellulose peak A_I. Peak (1) represents protein eluting in the void volume with 10mM potassium phosphate buffer. Addition of 1.6µM yeast tRNA resulted in elution of affinity-A_I enzyme (2). Peak (3) was eluted when buffer strength was increased to 300mM potassium phosphate.

methyltransferase activity seen in eluates of unstained gel slices is also shown in Fig. 3.

Enzyme characterization

Ion stimulation. Ionic conditions for maximal extent of methylation were determined with A_I enzyme and 0.64nM E. coli MRE tRNA. In the presence of this mixed population of tRNA, values of 0.89-0.94pmol methylated nucleoside/pmol tRNA were achieved with 5mM magnesium acetate, while 5mM putrescine

Table 2. Purification of 2mGII from T. pyriformis

	Total activity (units)	N^2-mG activity* (%/ units)	Total protein (mg)	Specific activity (units/mg protein)	Yield	Purification (fold)
High speed supernate	7300	28%/ 2044	4902	0.42	100%	1
DEAE Peak A	1666	36%/ 600	286	2.1	29.3%	5
Phosphocellulose A_I	770	99%/ 762	18.2	42.3	37.2%	73
Affinity A_I	128	100%/ 128	0.36	355.	6.2%	845

* N^2-mG activity is the sum of N^2-methylguanine and $N^2{}_2$-dimethyl guanine. % = percent of total methyltransferase activity.

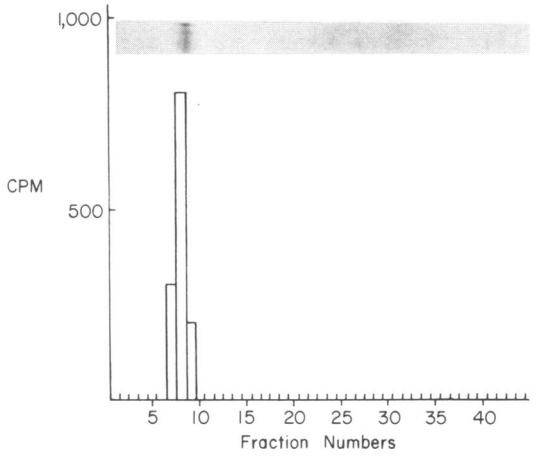

Fig.3 Non-denaturing electrophoresis of affinity A_I enzyme. Duplicate samples of enzyme were subjected to electrophoresis in 8% polyacrylamide. One lane was stained with Coonmassie blue (upper portion of figure) while the second was sectioned and gel sections assayed for methyltransferase activity .

Table 3. Substrate specificity

	EXTENT (pmol ^{14}C/ pmol tRNA)	% OF TOTAL AS: m^2G	$m^2{}_2G$
I. A_I Enzyme with			
a.tRNA containing G_{26}:			
$m^2{}_2G^-$ mutant	0.29	21%	79%
Leu_2	0.60	12%	88%
fMet	0.98	94%	6%
Ile	0.69	58%	42%
Ser_3	0.79	38%	62%
b.tRNA containing G_{10}:			
Phe	0.35	98%	2%
Val	0.46	98%	2%
II. Affinity A_I enzyme with			
a.tRNA containing G_{26}:			
Leu_2	0.45	12%	88%
Ser_3	0.38	4%	96%
b.tRNA containing G_{10}:			
Phe	<0.03	-	-
Val	<0.03	-	-

$m^2{}_2G^-$ tRNA was total tRNA prepared from the yeast trm 1 mutant; all other tRNAs were purified from E. coli.

produced 0.65pmol methylated nucleoside/pmol tRNA. Therefore, substrate specificity was examined in assay mixtures containing 5mM magnesium acetate.

Substrate specificity. The specificity of the partially purified A_I enzyme was examined using a variety of tRNAs containing guanine residues at position 26 and/or position 10 (Table 3). Total tRNA isolated from the yeast $m^2{}_2G^-$ mutant was methylated to an extent of 0.29 pmol/pmol tRNA; this RNA contains tRNA species lacking G at position 26 as well as species possessing a methylatable G_{26} as a result of 2mGII deficiency. Among the purified E. coli tRNAs tested, the best substrates for this enzyme were tRNAs containing a methylatable G_{26}; with these substrates extents of methylation ranged from 0.6-0.98 pmol methylated nucleoside/pmol tRNA. However, E. coli tRNAs lacking G_{26} which contained G_{10} residues were also substrates for the phosphocellulose-purified enzyme; methylation of $tRNA^{Phe}$ and $tRNA^{Val}$ resulted in 0.35-0.45pmol methylated product/pmol tRNA.

Analyses of the methylated products formed on G_{26}-containing tRNAs were consistent with results reported for mammalian 2mGII (12); a high proportion of dimethylguanine was found on all of these tRNAs except $tRNA^{fMet}$ (Table 3). The product formed on tRNAs with a methylatable G_{10} was m^2G, with <100 cpm in $m^2{}_2G$. These results suggest that methylation of $tRNA^{Phe}$ and $tRNA^{Val}$ was due to contamination of the 2mGII preparation with G_{10}-specific 2mGI activity.

The highly purified affinity-A_I enzyme showed little methylation of E.coli tRNAs lacking G_{26} (Table 3); methylation of $tRNA^{Phe}$ and $tRNA^{Val}$ was in the range of 1 pmol methyl/40 pmol tRNA (approximately 100cpm incorporated). Furthermore, a significant proportion of the ^{14}C-methylated product on $tRNA^{Phe}$ and $tRNA^{Val}$ was dimethylguanine, suggesting low level methylation of these tRNAs by 2mGII rather than 2mGI contamination. The affinity-A_I enzyme was therefore clearly superior in terms of purity. However, it consistently yielded less complete methylation of G_{26}-containing tRNAs than was obtained with the partially purified A_I enzyme preparations; the extent of methylation of $tRNA^{Leu}$ and $tRNA^{Ser}$ by

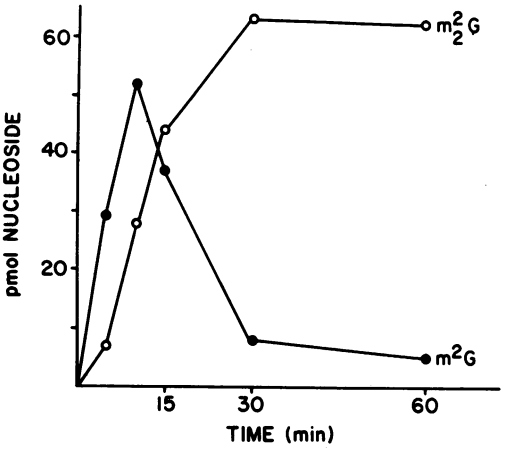

Fig.4 Product formation in E. coli $tRNA^{Leu}$ as a function of time. 0.64 units of A_I enzyme were incubated for varying amounts of time with limiting concentrations of tRNA (0.64µM). Substrate tRNA was hydrolyzed, the ^{14}C-methylated nucleosides resolved by HPLC and quantitated by liquid scintillation spectrometry.

affinity-A_I enzyme was 0.38-0.45pmol methyl/pmol tRNA (Table 3).

Formation of monomethyl- vs. dimethyl guanine:

The only products formed when A_I or affinity-A_I enzymes were incubated with G_{26}-containing E. coli substrates such as $tRNA^{Leu}$ or $tRNA^{Ser}$ were m^2G and $m^2{}_2G$. The ratio of monomethyl:dimethylguanine with 0.64units A_I enzyme and low tRNA concentrations (0.64µM) was time dependent (Fig 4). Incubations shorter than 15 min yielded m^2G as the major product while after 15 min $m^2{}_2G$ predominated. Under the same reaction conditions of limiting tRNA, the relative proportions of monomethyl and dimethylguanine were also affected by Mg ion concentration; however, reactions carried out with excess tRNA and limiting enzyme concentrations were relatively insensitive to Mg concentration (Fig 5). These results suggested that Mg ion was primarily interacting with the tRNA substrate; therefore, the effect of ionic pretreatment of tRNA on ionic requirements for methylation was examined.

Dialysis of tRNAs against buffers containing 10mM EDTA followed by exhaustive dialysis in the absence of EDTA (7)

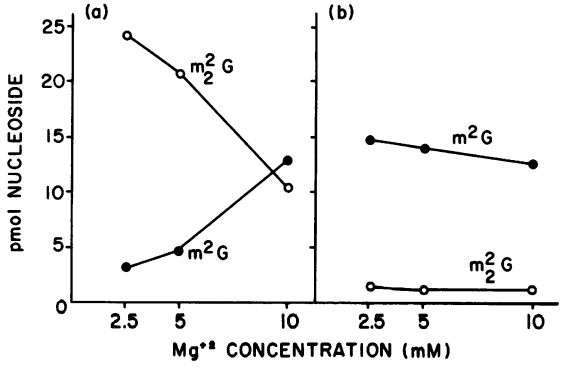

Fig.5 Influence of Mg^{2+} on methylation of E. coli $tRNA^{Leu}$ by A_I enzyme. (a) 0.64μM tRNA incubated for 20min with 0.64 units of A_I enzyme. (b) 3.2μM tRNA incubated for 20 min with 0.2 units of A_I enzyme.

resulted in an absolute requirement for cations in the methylation reaction. Reactions containing commercially prepared tRNAs which had not been exposed to EDTA showed variable but appreciable methylation in the absence of added cation. Cation-tRNA complexes were prepared by incubating EDTA-treated tRNAs with magnesium acetate or polyamines followed by dialysis to remove unbound cation as described previously (7). These tRNA complexes were fully active in supporting high levels of methylation in the absence of additional cation in the reaction mixture. Further analysis of the factors leading to monomethyl- vs. dimethylguanine formation was therefore carried out using cation-tRNA complexes.

The formation of dimethylguanine might be accomplished after a single association event involving the monomethylated product as an enzyme bound intermediate or in a two-step process requiring a separate association event for each methylation. These alternatives were examined in a series of experiments in which $m^2G/m^2{}_2G$ ratios were measured as a function of enzyme:tRNA ratio.

The effect of varying enzyme concentration in the presence of 0.64μM tRNA is shown in Fig.6. It is evident that a low enzyme/tRNA ratio favors m^2G formation while higher enzyme/tRNA ratios yield mostly $m^2{}_2G$. This is observed

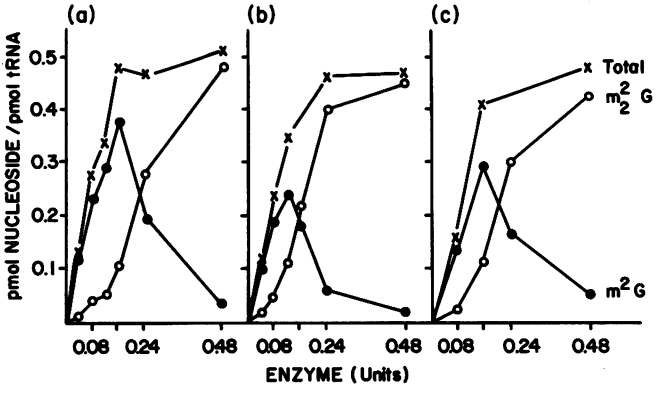

Fig.6 Effect of increasing A_I enzyme on methylation of E. coli tRNAs. All reactions were for 20min with 0.64μM tRNA; formation of N^2-methylguanine (●——●) and N^2_2-dimethylguanine (○——○) was determined by HPLC analysis of hydrolyzed tRNA. (a) Methylation of spermidine-treated $tRNA^{Leu}$. (b) Methylation of Mg-treated $tRNA^{Leu}$. (c) Methylation of Mg-treated $tRNA^{fMet}$.

whether the substrate is $tRNA^{fMet}$ or $tRNA^{Leu}$, and is independent of the cation complexed to the tRNA.

The results of reciprocal experiments, holding enzyme levels constant while varying tRNA concentration, are shown in Fig.7.

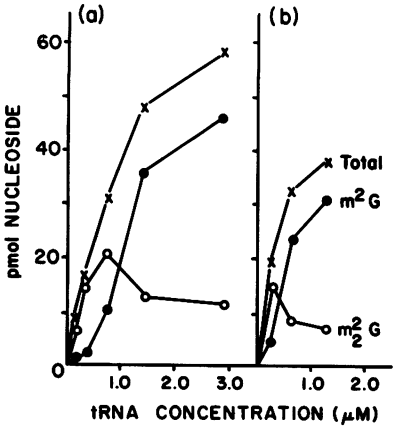

Fig.7. Influence of tRNA concentration on product formation. Varying amounts of Mg-treated $tRNA^{Leu}$ (a) and Mg-treated $tRNA^{fMet}$ (b) were incubated with 0.07 units of A_I enzyme with no additional ions. All reactions were 20min.

Once again, at lower enzyme/tRNA ratios the predominant product is monomethylguanine while at higher enzyme/tRNA ratios the amount of dimethylated guanine exceeds the monomethylated product. Both $tRNA^{fMet}$ and $tRNA^{Leu}$ displayed the same relative pattern; the major difference between the two tRNAs was that the crossover point at which monomethylguanine exceeded dimethylguanine occurred at 0.45µM $tRNA^{fMet}$ while with $tRNA^{Leu}$ this crossover required substrate concentrations closer to 1.0µM.

DISCUSSION

While methylation of the N^2-amino group of guanine occurs at several sites on eukaryotic tRNAs, most of these guanine modifications involve a single methyl addition to form m^2G. The methylation of G_{26} residues in eukaryotes is unique in that the product can be either a monomethylated or a dimethylated base. Dimethylation is by far the more common modification found at this site on sequenced cytoplasmic tRNAs, with m^2G_{26} reported thus far only for most initiator tRNAs (including Tetrahymena) and several $tRNA^{Val}$ species (21). Early studies of E. coli tRNA methylated with mammalian enzyme extracts showed that similar results are obtained in vitro; E. coli $tRNA^{fMet}$ was modified to m^2G while all other tRNAs containing a guanine at position 26 were modified to dimethylguanine (6,22,23).

Kuchino and Nishimura (22) first analyzed guanine-specific tRNA methylases from rat liver and demonstrated the presence of at least two enzymes: a G_{10}-specific enzyme which formed m^2G and G_{26}-specific activity which yielded both m^2G and $m^2{}_2G$. Spremulli et al. (24) and Kraus and Staehelin (12) succeeded in separating the G_{10} and G_{26} activities and discussed the possible scenarios for dimethylation of G_{26} residues. In one proposal, two enzymes are required for G_{26} dimethylation. These might function independently on specific tRNA species or sequentially; either mechanism could explain the observation that certain tRNA species are characteristically monomethylated while others are dimethylated. A second possibility involves only one G_{26} methyltransferase with mono-

vs. di-methylation as a function of the tRNA species.

The isolation of a yeast mutant lacking G_{26} methylating activity, the trm 1 strain of Phillips and Kjellin-Straby (25), has unfortunately not provided a definitive resolution to this question. While a mutation eliminating both methylations is consistent with the concept of a single enzyme, it cannot rule out a sequential process in which the enzyme lacking in the mutant supplies the m^2G required for a second enzyme.

Elucidation of the dimethylation reaction using a purified 2mGII has been hampered by the fact that this enzyme has been particularly difficult to obtain in high purity from the customary mammalian sources. The observation that Tetrahymena pyriformis contains relatively high levels of the tRNA methyltransferases (26), prompted us to examine this ciliated protozoan as an enzyme source. The most highly purified 2mGII preparations obtained from Tetrahymena appear to be homogeneous on non-denaturing polyacrylamide gels. The protein band contains both m^2G and $m^2{}_2G$-forming capability, thus demonstrating that 2mGII is a single enzyme with dimethylating ability. Although the less pure enzyme preparation catalyzes virtually complete methylation of guanine on those E. coli tRNAs containing G_{26}, extents of methylation with the homogeneous enzyme are lower, presumably due to instability of the highly purified enzyme.

The mechanism by which $m^2{}_2G$ is formed might involve a single tRNA-enzyme association event resulting in $m^2{}_2G$ formation or, alternatively, dissociation might occur between successive methylations. Initial time course experiments under tRNA-limiting conditions indicated that the latter alternative was more likely. If dimethylguanine formation proceeds after a single binding event the $m^2G/m^2{}_2G$ ratio should remain constant over time. We observed, however, that monomethylguanine predominates at early times and that dimethylguanine accumulates only at later time periods. While it was possible that addition of the second methyl group is markedly slower than the first methylation, the significant lag suggested that tRNA dissociated between successive methylations. If so, the substrate for

dimethylguanine formation would be the pool of free tRNAs containing m^2G.

This was explored in a series of experiments in which the enzyme:tRNA ratio was varied. If no enzyme-tRNA dissociation occurs between m^2G and $m^2{}_2G$ formation, the pool of m^2G-containing tRNA intermediates should be bound to enzyme. In this case, little difference in monomethylguanine: dimethylguanine ratios would be observed between tRNA-limiting and tRNA-excess conditions. However, if dissociation occurs, the pool of m^2G-containing tRNAs will be diluted by free unmethylated tRNA molecules. The degree of dilution would then determine the kinetics of dimethylguanine formation.

Reactions with the Tetrahymena A_I enzyme under tRNA limiting conditions favored dimethylguanine formation, even at short reaction times. However, with high tRNA:enzyme ratios, there was a delay in dimethylguanine formation. This confirms that enzyme-tRNA dissociation occurs between successive methylations. Remaining to be determined is whether dissociation is necessary in order to discharge S-adenosylhomocysteine and bind a second S-adenosylmethionine, or for rotation of the N^2-monomethyl group, or both.

The purified 2mGII from Tetrahymena can be compared with the (less purified) 2mGII activities isolated from normal and neoplastic rat tissues. Brunke and Leboy (11) reported molecular weights for liver, mammary gland and mammary adenocarcinoma in the range of 180-200kD for rat 2mGII. The Tetrahymena 2mGII enzyme appears to have a similar molecular weight.

Like the mammalian 2mGII enzyme preparations, the Tetrahymena enzyme will methylate a variety of *E. coli* tRNAs containing a guanine residue at position 26. The predominant product with mammalian 2mGII enzyme and most *E. coli* tRNAs is dimethylguanine; however, *in vitro* methylation of *E. coli* $tRNA^{fMet}$ results in monomethylguanine at position 26 (12). In contrast,reactions with Tetrahymena 2mGII and ion-treated *E. coli* $tRNA^{fMet}$ yielded significant amounts of dimethylguanine; indeed, at low tRNA concentrations dimethylguanine was the predominant product. This prompted us to re-examine methylguanine formation by mammalian 2mGII (using a 25-55%

ammonium sulfate fraction from rat liver high speed supernatant) with E. coli $tRNA^{fMet}$ pretreated with magnesium ions. Approximately 30% of the methylated guanine product was dimethylguanine (unpublished observations). These results suggest that, while the Tetrahymena enzyme yields a higher proportion of dimethylguanine than does mammalian 2mGII, for both of these enzymes the $m^2G:m^2{}_2G$ ratio on initiator tRNA is influenced by cation binding to the tRNA.

While the substrate requirement of Tetrahymena 2mGII is a methylatable G_{26} residue, low level methylation (0.02-0.03 pmol/pmol tRNA) of several tRNAs lacking G_{26} was observed. Analysis of the methylated products revealed both monomethyl- and dimethylguanine, suggesting an aberrant methylation was occurring. This warrants further study, since mammalian tumor-derived 2mGII (but not that from non-neoplastic tissues) causes low level methylation of tRNAs lacking G_{26} (11). We are therefore currently using polyclonal and monoclonal antibodies raised against Tetrahymena 2mGII to compare the properties of the protozoan and mammalian enzymes.

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