Interaction of tRNA with tRNA (Guanosine-1)methyltransferase: Binding Specificity Determinants Involve the Dinucleotide $G^{36}pG^{37}$ and Tertiary Structure†

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Received January 23, 1997; Revised Manuscript Received April 17, 1997⊗

ABSTRACT: The sequence $G^{37}pG^{36}$ is present in all tRNA species recognized and methylated by the *Escherichia coli* modification enzyme tRNA (guanosine-1)methyltransferase. We have examined whether this dinucleotide sequence provides the base specific recognition signal for this enzyme and have assessed the role of the remaining tRNA in recognition. *E. coli* $tRNA^{His}$ and yeast $tRNA^{Asp}$ were substituted with G at positions 36 and 37 and were found to be excellent substrates for methylation. This suggested that the general tRNA structure can be specifically bound by the enzyme. In addition, heterologous tRNA species including fully modified $tRNA_1^{Leu}$ are excellent inhibitors of $tRNA_1^{Leu}$ transcript methylation. Analyses of structural variants of yeast $tRNA^{Asp}$ and *E. coli* $tRNA_1^{Leu}$ demonstrate clearly that the core tertiary structures of tRNA are required for recognition and that G^{37} must be in the correct position in space relative to important contacts elsewhere in the molecule. This latter conclusion was reached because the addition of one to three stacked base pairs in the anticodon stem of $tRNA_1^{Leu}$ dramatically alters activity. In this case, the G37 base is rotated away from the correct position in space relative to other tRNA contact sites. The acceptor stem structure is required for optimal activity since deletion of three or five base pairs is detrimental to activity; however, specific base sequence may not be important because (i) the addition of three stacked base pairs of different sequence had little effect on activity and (ii) heterologous tRNAs with little or no sequence homology in the acceptor stem are excellent substrates. Both poly G and GpG are potent and specific inhibitors of enzyme activity and are minimal substrates which can be methylated, forming m^1G. Taken together, these studies suggest that 1MGT can bind the general tRNA structure and that the crucial base-pair contacts are G37 and G36.

The importance of RNA modification is underscored by the fact that about 1% of the *Escherichia coli* genome encodes tRNA modification enzymes (*1*). These enzymes are essential for the correct function of cellular RNA's such as tRNA in protein synthesis (*2*). The enzyme tRNA (guanosine-1)methyltransferase (1MGT) modifies a subset of tRNA's which all recognize only codons beginning with C such as those for leucine, proline, and arginine. Therefore in these tRNA's positions 36 and 37 constitute the sequence GpG. It has been shown that methylation at G37 prevents translational frameshifting and that in strains of *Salmonella typhimurium* lacking m^1G37 in $tRNA^{Pro}$, quadruplet translocation occurs at specified codon sites (*3*, *4*). 1MGT is the product of the *trmD* gene which is encoded within an operon containing four genes (*5*−*7*). It is co-transcribed with two ribosomal protein encoding genes (*rpsP* and *rplS*), and a ribosomal maturation protein gene *rimM*. Marked translational polarity is seen in the expression of *trmD* operon proteins. For example, the relative expression of the trmD protein is 40-fold less than for the ribosomal protein genes. This may be due to a poor Shine-Dalgarno sequence and possible secondary/tertiary structure in operon mRNA (*8*). This results in a very low level of expression of *trmD* which, interestingly, is invariant with growth rate even though mRNA levels increase as a function of growth rate.

Given that *E. coli* may have at least 29 different modified bases which are synthesized by about 45 enzymes (*9*), it should not be surprising if a great diversity of recognition mechanisms is demonstrated. Indeed, modifications are to be found in several domains of tRNA located in remarkably different RNA tertiary structural milieu. For example, U residues must be pseudo-uridylated *in situ* in various structural contexts to include helical regions in the anticodon stem, and in loop structures such as the ubiquitous TYCG sequence in the T loop of tRNA. Other examples include a plethora of methylation reactions in virtually all regions of tRNA and hypermodifications of the anticodon loop. In these cases the local RNA tertiary structure is undoubtedly diverse and it is likely that proteins must have evolved to fit divergent RNA topologies if appropriate contacts are to be made during catalysis. Recent studies suggest that modification enzymes may indeed display considerable variation in complexity with regard to RNA structural determinants recognized. For example, in some cases nucleotide residues required for recognition lie close to the site(s) for modification as is seen with Y biosynthesis at position 37 of yeast $tRNA^{Phe}$, and in the case of methylation of U54 in most

† Supported by Grant MCB-9408313 from the National Science Foundation and by the C.N.R.S.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

S0006-2960(97)00153-0 CCC: $14.00

tRNA's (*10*). In the case of U54 methylation, a simple stem−loop structure is sufficient for recognition. In other cases the general tertiary structure of tRNA is required for recognition as is the case for the synthesis of ψ35 in *Arabidopsis* $tRNA^{Tyr}$ (*11*) , $^1mG^{37}$ in *E. coli* (*12*), and the dimethylation of G26 in yeast $tRNA^{asp}$ (*13, 14*). The Grosjean laboratory recently examined the long-range effects of mutations in tRNA on modification of residues in other places in the molecule. In particular, D stem and variable loop substitutions affect anticodon loop modifications including M1G37, Q34/manQ34,40 (*14*). Little is known about the sequence and interdependence of tRNA modification, but it is likely that it is an efficient and vectorial process. Given the apparent diversity of enzymes which modify RNA, it will be interesting and important to determine what general and specific features exist in this class of RNA binding proteins which enable these molecules to recognize remarkably diverse RNA structures in the highly structured tRNA molecule. Work is just beginning concerning exact RNA structures required for modification enzyme binding and catalysis, and virtually nothing is known about protein structures required for recognition.

In this communication we have shown that $G^{36}pG^{37}$ is a crucial identity element and if placed in the context of a number of heterologous tRNA species yields an excellent substrate. Moreover, GpG or poly G alone can serve as substrates albeit with low efficiency for the enzyme, but to serve efficiently this sequence must be positioned correctly in space with respect to the rest of the tRNA structure.

MATERIALS AND METHODS

Reagents. All nucleotides and oligonucleotides including GpG were obtained from Sigma. T7 RNA polymerase was purified in quantity as described elsewhere (*15*). Restriction endonucleases were obtained from New England Biolabs or BRL and utilized with protocols provided by the manufacturers.

Enzyme Purification. Enzyme was purified by two methods for these studies. Initially, enzyme was prepared using an *E. coli* system previously described wherein the *trmD* gene was inserted into appropriate expression vectors under the control of a T7 promoter. In this case PCR techniques were used such that an optimal Shine-Dalgarno sequence was inserted in the appropriate position for expression (*16*). The strain containing this construction was designated pHR118/HMS174(DE3). Typically 12−14 L of cells were harvested at late-log phase from LB medium containing ampicillin 2−3 h after adding 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside). Cells were washed then resuspended in 50 mL of buffer A [25 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 0.1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 10% ethylene glycol or glycerol]. Cells were then disrupted at 16 000 pounds per square inch in an Aminco French pressure cell. Next, DNase (2.5 mg) was added and DNA digested at 37 °C for 15 min. The extract was clarified by centrifugation at 27000*g* for 1 h, then brought to a concentration of 0.6 M $(NH_4)_2SO_4$ before ultracentrifugation at 300000*g* to remove ribosomes. Following dialysis in Buffer A, the supernatant was brought to 0.05 M NaCl and then applied to a DEAE-Sephacel column (2.5 × 25 cm) equilibrated in the same buffer. Following extensive washing with the low salt buffer, proteins were eluted with a 500 mL increasing salt gradient up to 0.5 M NaCl in Buffer A. Enzyme activity was detected as previously described (*17*). Pooled fractions containing enzyme activity were dialyzed in Buffer B (10 mM potassium phosphate buffer, pH 7.0, 0.1 mM DTT, 10% ethylene glycol v/v) and then applied to a Heparin Sepharose Column (2.5 × 7 cm) equilibrated in buffer B. A 200 mL gradient of increasing salt from 0 to 0.5 M NaCl eluted bound proteins, and pooled fractions containing enzyme were dialyzed in buffer C (20 mM potassium phosphate, pH 7.0, 0.1 mM DTT, and 10% ethylene glycol). Protein was next applied to a Bio-Gel HTP hydroxyapatite column (2 × 6 cm). Enzyme does not stick under these conditions and elutes in very pure form (at least 95% pure) in Buffer C. In order to facilitate enzyme purification for these and other studies, a "his-tagged" enzyme was prepared. This was done using vectors and standard cloning procedures as outlined by the Qiagen Corporation. The *trmD* gene was inserted into the Qiagen commercial vector pQE30 using the *Sal*I and *Bam*HI sites to achieve directional cloning. Synthetic oligos (primer for amino end, 5′ AAAGACGGATCCATGTGGATT; PCR primer for carboxy end, 5′ TCCTGGGTCGACTGATATCTCGGGGGC) were used as primers for PCR as previously described (*16*) in order to tailor DNA ends for cloning with a *Sal*I site defining the 5′ end of the *trmD* gene insert. Following the insertion of the resulting PCR fragment into the PQE30 (Qiagen Corporation) vector, the trmD initiation codon immediately follows the *Sal* sequence of pQE30, and the stop codon immediately follows the *Bam* site of that vector. The resulting recombinant plasmid was transformed into *E. coli* strain MJ109. The resultant *trmD* gene his-tagged derivative was then induced using IPTG in 4 L of LB medium containing ampicillin. Induction was initiated on mid-log cells and continued for 3−4 h. Cells were harvested and washed in 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, and then disrupted in the French pressure cell as outlined above. Crude extracts were spun at 25000*g* for 1 h, and the supernatant was loaded directly onto a 2.5 × 7 cm column of $Ni^{2+}NTA^-$ agarose (Qiagen) after the addition of 10 mM imidiazole. Enzyme was eluted with a linear gradient of 10 mM to 0.5 M imidiazole in 50 mM sodium phosphate, pH 8.0, 0.3 M NaCl, and 10% glycerol v/v. Next, enzyme was loaded onto a ConSep LC100 monoQ FPLC column following dialysis with Buffer A containing 0.05 M NaCl. Protein was eluted with a linearly increasing NaCl gradient to 0.5 M. Enzyme at this point was at least 95% pure. The typical yield of enzyme was approximately 100 mg per 4 L of induced, late-log cells. The kinetic properties of these two enzymes were compared and shown to be very comparable and K_m/V_{max} values were essentially identical. The specific activities of wild type and his-tagged enzyme were 12.4 and 15.2 nmol/min/mg of protein, respectively. His-tagged enzyme was used for most of these studies. All mutant tRNA studies were carried out with equal units of his-tagged enzyme.

Preparation of Native and Transcript tRNA. Native *E. coli* $tRNA_1^{Leu}$, $tRNA_4^{Leu}$, and $tRNA^{His}$ were provided by Dr. Brian Reid (Department of Chemistry, University of Washington, Seattle). Synthetic $tRNA_1^{Leu}$ and "hairpin" $tRNA_1^{Leu}$, $tRNA^{His}$, and $tRNA^{Asp}$ substituted with G at positions 36 or 37 were prepared via "runoff" *in vitro* transcription using T7 RNA polymerase as previously outlined (*12*). In the case of $tRNA^{His}$, runoff transcription was carried out using

templates cleaved with *Bam*HI rather than *Bst*NI which was used for all others. This resulted in a transcript with extra bases at the 3′ terminus (Figure 3). DNA synthetic oligos for wild type and mutant yeast $tRNA^{Asp}$ were phosphorylated, ligated, and then cloned using the vector pUC118 as previously outlined (*12*, *16*). Synthetic tRNA was prepared *in vitro* as previously described (*12*). Typical transcription mixtures contained 40 mM Tris-HCl, pH 8.0, 22 mM $MgCl_2$, 25 mM dithiothreitol (DTT), 2 mM spermidine, 4 mM each of the nucleotide triphosphates (NTP's), 40 mM GTP, 50 μg of pure T7 RNA polymerase, and 50 μg of *Bst*NI cut template. Typical transcriptions were carried out for 4 h, and following phenol extraction and ethanol precipitation transcripts were purified using HPLC through a TSK G2000 molecular sieve column equilibrated with 0.2 M sodium acetate, pH 6.0, with 1% methanol v/v.

GpG and polyG Methylation and Enzyme Assay. In general, *in vitro* methylation was determined in 50 μL reaction mixtures essentially as outlined elsewhere (*12*, *17*). Typically, 5 μg of pure enzyme (specific activity 15.2 nmol/min/mg of protein) was employed unless otherwise specified in 4-min assays. In the case of competition studies, competing RNA was added before $tRNA_1^{Leu}$ transcript.

GpG (Sigma, 10 mM) and polyG (Sigma, approximately 3 mM assuming an average length of 400 nucleotides) were methylated for 1.5 h. The reaction mixtures contained, in 100 μL, 120 μM [^{14}C]AdoMet (55 mCi/mM), 0.1 M Tris-HCl, pH 8.0, 0.1 mM EDTA, 6 mM $MgCl_2$, 24 mM NH_4Cl, and 24 μg of bovine serum albumin. Reactions were started by the addition of 5 μg of pure enzyme. Following incubation, reaction mixtures were ethanol precipitated, lyophilized, and dissolved in 20 μL of 0.3 M KOH. Hydrolysis was carried out for 12 h at 37 °C.

Analysis of Methylated GpG and PolyG. Two μL of these preparations were then spotted onto DC-Fertigplatten Cellulose thin-layer plates and submitted to two-dimensional thin-layer chromatography. The first-dimension solvent consisted of an isobutyric acid/ammonia/water mixture (66:1:33 v/v/v), and the second 0.1 M phosphate buffer, pH 6.8, containing 2% *n*-propanol (v/v) and 60 g % ammonium sulfate. Labeled nucleotides were localized by autoradiography.

Inhibition Studies. Increasing concentrations of purified heterologous tRNA species to include $tRNA_4^{Leu}$, $tRNA^{His}$, and native, fully modified $tRNA_1^{Leu}$ (2.71−51.85 μM) were incubated with 1MGT in the presence of a fixed concentration of $tRNA_1^{Leu}$ *in vitro* transcript (0.34 μM in the case of minihelix inhibition or 1.36 μM for all other experiments). Assay procedures were as outlined previously. In control experiments it was shown that all inhibiting RNA species were not detectably methylated during the time course of the assay. In experiments using poly G and GpG, K_i values were determined using increasing concentrations of poly G, GpG, and other nucleotides up to 20 mM. tRNA concentrations used in all cases were 0.72 or 1.44 μM.

RESULTS

Purification and Characterization of tRNA (Guanosine-1)methyltransferase

Enzyme Isolation and Purification. A major problem in studying modifying enzymes is the availability of enzymes in a pure enough form and in sufficient quantities for analysis. Initially we developed an improved procedure similar to that of others (*17*) for lMGT purification. We were able to produce about 20 mg of over 90% pure enzyme from 3.5 g of crude protein. Strains and modified plasmids used here have been described elsewhere (*17*, *16*). In an attempt to further expedite the production of large quantities of enzyme we have prepared a his-tagged form of the enzyme as described in Materials and Methods. Typically we can prepare at least 100 mg of enzyme from only a few liters of cells very quickly. Enzyme prepared in this manner displays kinetic parameters comparable with those of native enzyme as pointed out in Materials and Methods. Most of the studies to be described were done using his-tagged preparations. Figure 1 shows the relative purity of typical preparations prepared in both ways. Interestingly we consistently observe that purified 1MGT runs anomalously slowly in SDS gels since it migrates slightly slower than the 31 kDa molecular weight standard. A molecular weight of approximately 28 800 can be predicted from the known amino acid sequence. This is true as well for his-tagged enzyme although it can be seen that as predicted it migrates more slowly than native enzyme in view of the extra histidine-rich residues placed at the amino terminus. Polyclonal antibody provided by Dr. Glen Bjork prepared against purified enzyme (University of Umeå, Umeå, Sweden) reacts strongly and specifically with both purified preparations (data not shown).

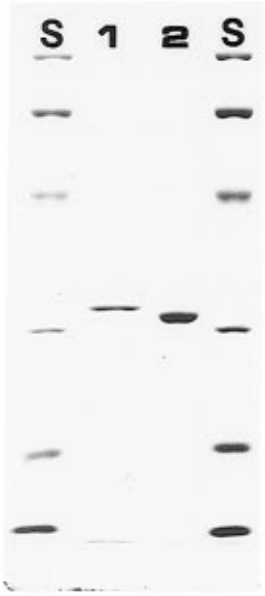

FIGURE 1: Purity and molecular weights for normal and his-tagged 1MGT as judged by SDS−polyacrylamide gel electrophoresis. Lanes from left to right: his-tagged enzyme (1), normal enzyme (2). Molecular weights for protein standards (S) which flank lanes 1 and 2 from top to bottom: 97 400, 66 200, 45 000, 31 000, 21 500, 14 400. 10% SDS linear gels were employed.

Kinetic Properties. It was important to determine if tRNA enzyme interactions could be studied in the absence of the methyl donor *S*-adenosylmethionine (AdoMet), and if AdoMet affects the affinity of tRNA for the enzyme or *vice versa*. Therefore, kinetic experiments were performed to explore the nature of binding site interactions. For these experiments $tRNA_1^{Leu}$ *in vitro* transcripts were employed. The data shown in Figure 2 are consistent with a *random-sequential* mechanism whereby tRNA K_m values are unaffected in tRNA titrations at various AdoMet concentrations. In addition, it can be seen that activity is strongly and competitively

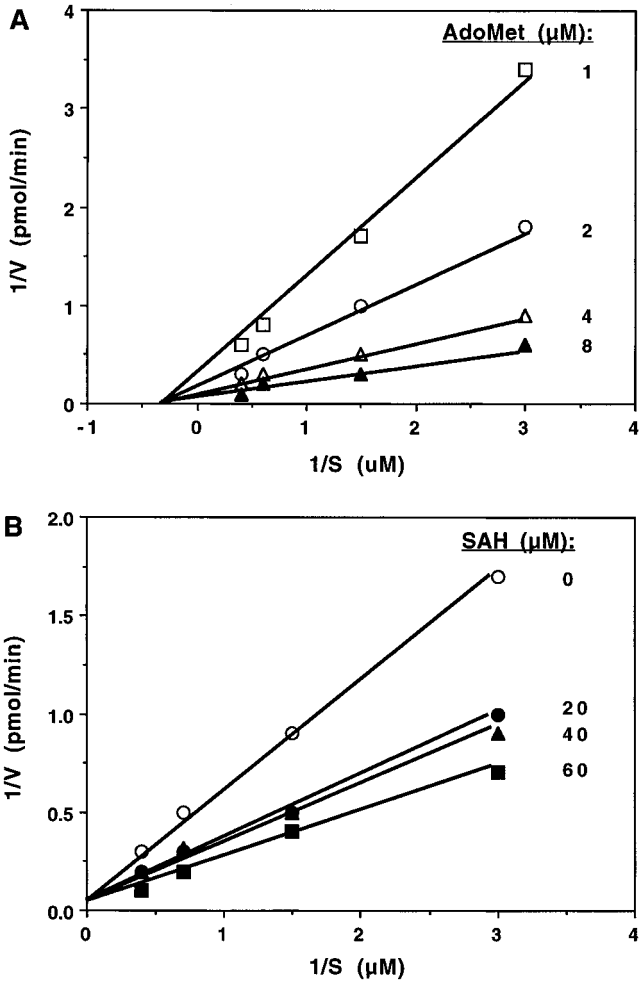

FIGURE 2: Kinetic analysis of $tRNA_1^{Leu}$ *in vitro* transcript methylation by 1MGT. (A) Methylation of increasing concentrations of tRNA (0.34−2.72 μM) in the presence of 1, 2, 4, and 8 μM AdoMet. (B) Methylation of increasing concentrations of tRNA 0.34−2.72 μM) in a fixed concentration of AdoMet (50 μM) and increasing concentrations of *S*-adenosyl homocysteine (SAH) of 20, 40, then 60 μM.

inhibited by the product of the reaction *S*-adenosylhomocysteine.

Methylation of Canonical tRNA Substrates.

In our previous studies (*12*) it was shown that the entire tRNA structure containing G residues at positions 36 and 37 was required for optimal catalytic activity. It occurred to us that any tRNA species structure containing G residues at position 36 and 37 might serve as a substrate for the enzyme. If this were true, then primary RNA contacts may in large part be the phospho-sugar backbone of tRNA or perhaps the invariant bases of tRNA. In order to test this idea we introduced G residues at position 36 into yeast $tRNA^{Asp}$ and *E.coli* $tRNA^{His}$ *in vitro* transcripts. These tRNA species show little relative homology to $tRNA_1^{Leu}$, and many base residues are different except for the invariant bases of tRNA (*18*). Both were found to be excellent substrates for 1MGT, as shown in Figure 3. It should be pointed out that in the case of $tRNA^{His}$, it was impossible to introduce a *Bst*NI site due to the primary structure of the tRNA. Therefore cleavage was carried out with *Bam*HI which yielded tRNA with extra nucleotides following runoff transcription (Ext-$tRNA^{His}$ G36,G37). As a control, $tRNA_1^{Leu}$ template was transcribed following cleavage with the same enzyme (pUTL4E). This yielded transcripts (Ext-$tRNA_1^{Leu}$) with the same extra nucleotides at the 3′ end. Kinetic analyses demonstrate that the addition of extra nucleotides to the 3′ end of $tRNA_1^{Leu}$ transcript has little effect on catalytic activity (V_{max} = 28 pmol/min, K_m = 3.6 μM) as compared to control $tRNA_1^{Leu}$ transcript (V_{max} = 33 pmol/min, K_m = 3.6 μM, Table 1). These results indicate that the tRNA 3′ terminus is not involved in enzyme recognition.

Interaction of 1MGT with Heterologous Substrates. These results given above support the idea that enzyme can bind a general tRNA structure. If this is true then it can be predicted that the anticodon hairpin lacking the major structural features of tRNA (Figure 4) would not be bound and therefore be a poor substrate, even if an appropriate methyl-

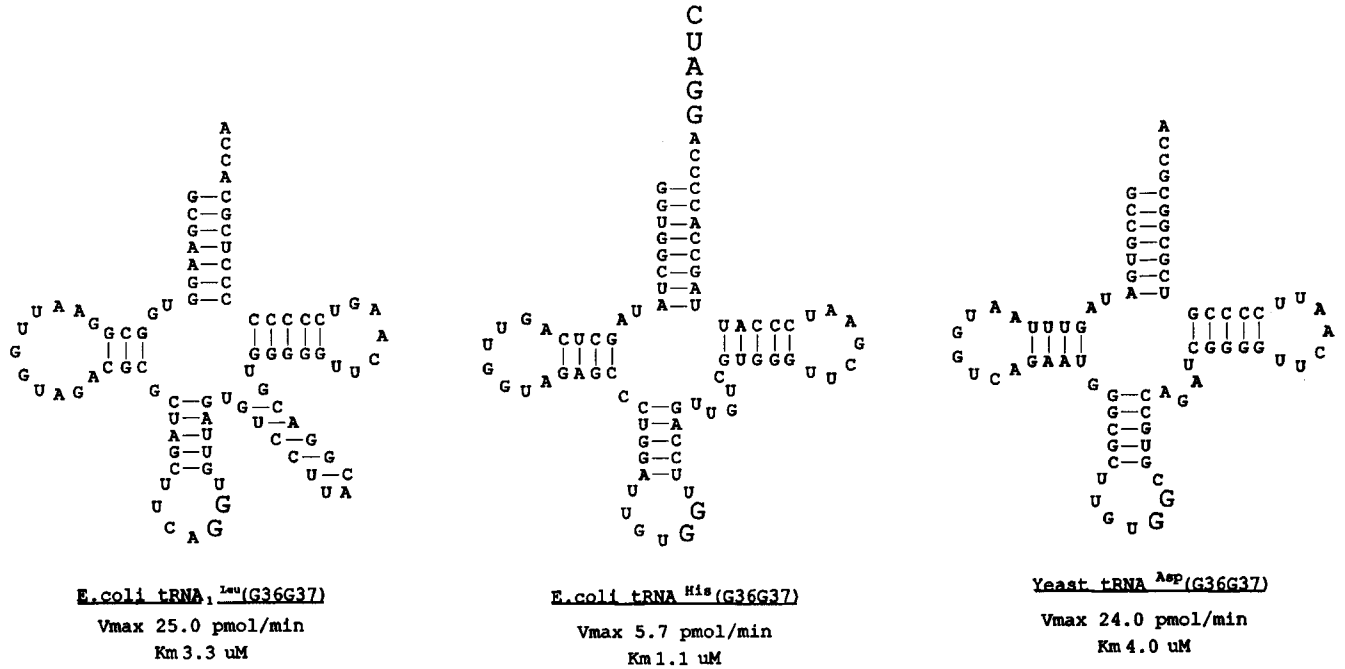

FIGURE 3: Efficient, heterologous tRNA substrates for 1MGT. (A) Wild-type $tRNA_1^{Leu}$; (B) *E. coli* $tRNA^{His}$; (C) yeast $tRNA^{Asp}$. All represent *in vitro* transcripts.

Table 1: Apparent Kinetic Values of 1GMT Substrates

figure	transcript	V_{max} (pmol/min)	K_m (μM)	V_{max}/K_m	V_{max}/K_m relative
	canonical tRNAs				
3A/6A	*E. coli* $tRNA_1^{Leu}$ (pUTL4)	25.0	3.3	7.6	1.00
	E. coli $tRNA_1^{Leu}$ (pUTL4E)	28.0	3.6	7.8	1.03
3B	*E. coli* $tRNA^{His}$	5.7	1.1	5.2	0.68
3C	yeast $tRNA^{Asp}$ G36G37	24.0	4.0	6.0	0.79
	architectural variants				
	E. coli $tRNA_1^{Leu}$ derivatives				
4A	hairpin structure	5.0	101.0	0.1	0.01
6B	+3 bp in anticodon stem	0.4	3.9	0.1	0.01
6C	+1 bp in anticodon stem	1.7	3.3	0.5	0.07
6D	U32>A32	2.1	5.0	0.4	0.05
6E	U32>C32	12.5	3.3	3.8	0.50
6F	+3 Gs in anticodon loop		inactive		
6G	−3 bp in acceptor stem	2.5	6.3	0.4	0.05
6H	−5 bp in acceptor stem	2.5	6.3	0.4	0.05
6I	+3 bp in acceptor stem	25.0	10.0	2.5	0.33
	yeast $tRNA^{Asp}$ G36G37 derivatives				
7A	disruption of A14:U8/A15:U48	0.4	3.9	0.1	0.02
7B	disruption as in 7A/compensation	0.6	3.1	0.2	0.03
7C	disruption of T/D loop interaction	25.0	16.6	1.5	0.25
7D	disruption as in 7C/compensation	25.0	10.0	2.5	0.42

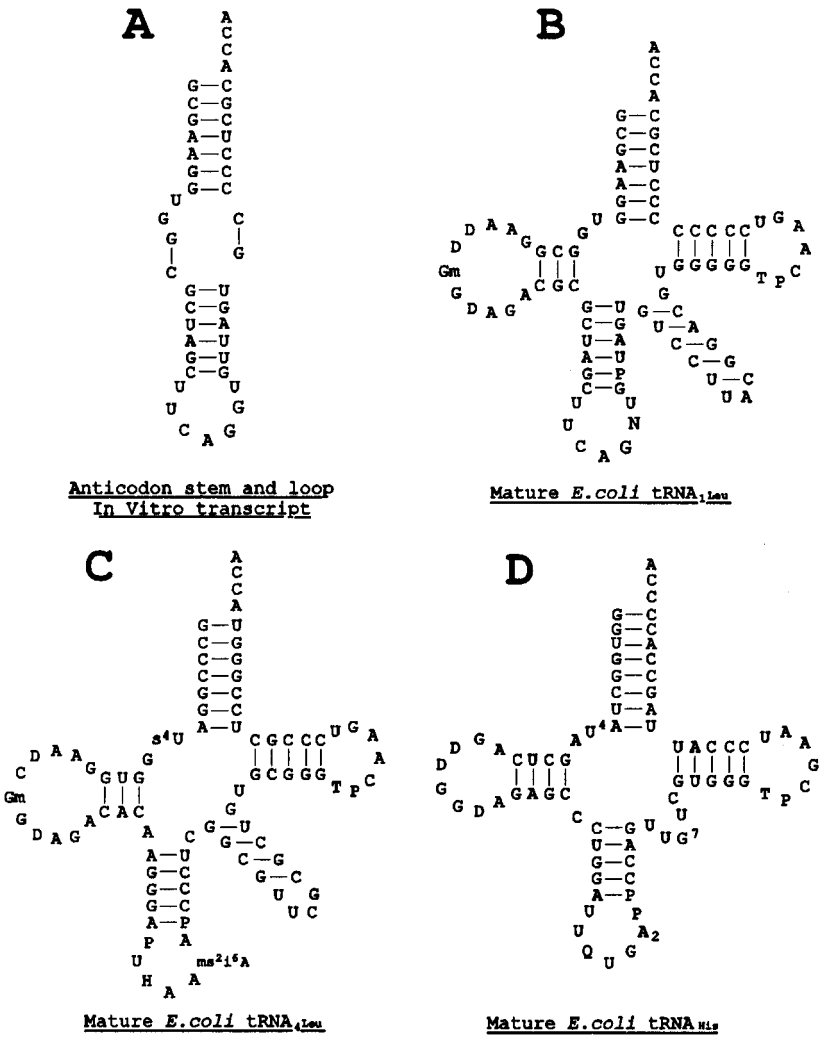

FIGURE 4: Sequence of tRNA tested for the capacity to inhibit the methylation of wild-type transcript. (A) $tRNA_1^{Leu}$ hairpin *in vitro* transcript; (B) mature $tRNA_1^{Leu}$; (C) mature $tRNA_4^{Leu}$; (D) mature $tRNA^{His}$. Note that mature tRNAs are completely modified. Abbreviations used in these structures: Gm = 2′-*O*-methylguanosine, *D* = dihydrouridine, P = pseudouridine, T = 5-methyluridine, s4U = 4-thiouridine, H = unknown modified adenosine, ms2i6A = 2-methylthio-N^6-isopentenyladenosine, A2 = 2-methyladenosine, G7 = 7-methylguanosine, N = 1-methylguanosine, Q = queuosine.

ation site in the anticodon loop were provided. That this is indeed the case is shown in Table 1. Note that the K_m value is about 30-fold more than that of $tRNA_1^{Leu}$. To determine whether this K_m value indeed represents poor binding of the $tRNA_1^{Leu}$ hairpin to the enzyme, an inhibition study was done in which increasing amounts of the hairpin were mixed with

Table 2: Inhibition Kinetics for Heterologous tRNAs, Poly G, GpG, and Other Dinucleotides[a]

inhibitor	I_{50} (μM)	K_i (mM)
mature $tRNA_1^{Leu}$	61.2	
mature $tRNA_4^{Leu}$	54.4	
mature $tRNA^{His}$	95.2	
GpG		6
polyG		2
polyU		-
GMP		-
ApG		-
GpU		-
GpC		-
GpA		-

[a] In all cases the $tRNA_1^{Leu}$ substrate was at 0.28 μM. In the case of poly G, GpG, and other dinucleotides, concentrations of 5, 10, 15, and 20 mM were added to separate assays done in triplicate. The I_{50} was extrapolated from standard activity plots. In the case of all dinucleotides except for GpG no detectable inhibition was detected. In the case of the minihelix no detectable competition was seen up to a 30-fold molar excess.

a constant amount of wild type tRNA transcript (pUTL4) and methylation measured. Under the conditions used, the competing hairpin was not measurably methylated. Therefore, any decrease in the initial velocity of methylation of wild type transcript could be attributed to an inhibition of substrate binding. The hairpin $tRNA_1^{Leu}$ derivative has poor affinity for the enzyme since no inhibition of wild type tRNA methylation was detected up to the addition of a 30-fold molar excess of competing RNA. This is consistent with the large K_m value obtained for the hairpin (Table 1), and shows that a simple stem and loop structure is not sufficient for optimal binding by this enzyme. We have shown that both $tRNA^{Asp}$ $G^{36}G^{37}$ and $tRNA^{His}$ $G^{36}G^{37}$ are excellent substrates. Therefore, it would be important to determine if G^{37} and G^{36} were essential for binding, or if any tRNA structure might bind the enzyme. This was tested by inhibition assays performed with two heterologous *E. coli* tRNA species, namely, mature $tRNA_4^{Leu}$ and $tRNA^{His}$. Sequences and modifications for these tRNA species are given in Figure 4 as reported elsewhere (*18*).

These tRNA's are not methylated by 1MGT since they are modified at position G37. In these studies, the results of which are given in Table 2, increasing concentrations of inhibiting tRNA were incubated with enzyme prior to assay. A 50% inhibition (I_{50}) was observed with 54.4 and 95.2 μM (Table 2). This indicates that indeed m^1G methyltransferase binds diverse tRNA species even in the absence of G residues at positions 36 and 37. On the basis of these results we propose that G^{37} is required for efficient binding since K_m values suggest much better binding for $tRNA_1^{Leu}$ transcript (K_m is approximately 3 μM). Table 2 shows the results of a competition study between mature $tRNA_1^{Leu}$, $tRNA_4^{Leu}$, $tRNA^{His}$, and synthetic $tRNA_1^{Leu}$ from pUTL4. Here it can be seen that modified $tRNA_1^{Leu}$, $tRNA_4^{Leu}$, and $tRNA^{His}$ reduce methylation of $tRNA_1^{Leu}$ transcript by 50% at concentrations of 61.2, 54.4, and 95.2 μM, respectively. These results suggest that the product of the reaction, mature $tRNA_1^{Leu}$, has an affinity for enzyme comparable to levels of other tRNA species.

Importantly, it appears that other $tRNA_1^{Leu}$ modifications may not affect binding of our enzyme to tRNA, since the reported K_m of bulk tRNA from *trmD1* mutant cells hypomodified only at position G37 is 20 μM (*17*). This value compares well with 3.25 μM for $tRNA_1^{Leu}$ *in vitro* transcript (*12*) particularly when considering that bulk tRNA contains other nonspecific tRNA species which are inhibitory and other tRNA substrates which may have higher K_m values.

Methylation of Minimalist Substrates

The above results further suggest that the active site of the enzyme may bind the dinucleotide sequence GpG which therefore might be the most minimal substrate for the enzyme. In order to test this hypothesis we attempted to (a) determine if the dinucleotide GpG and poly G can inhibit enzyme activity and (b) determine directly if these molecules can be methylated in the one position of G residues. Table 2 shows that poly G inhibits the methylation of $tRNA_1^{Leu}$ transcript with a K_i of 2 mM. Inhibition appears to be G specific since poly U gave little or no inhibition up to 6 mM. The K_i for GpG was 6 mM, whereas no detectable inhibition was detected in the presence of 5 mM of ApG, GpC, and GpU. Next, we determined if in fact GpG and poly G could be methylated by the enzyme since it was possible that these compounds might bind and block methylation but yet not possess tRNA structures which are required for methylation by bound AdoMet. In order to test this idea, we attempted to label these compounds enzymatically with [^{14}C]AdoMet, digest with alkali, and then separate via two-dimensional thin-layer chromatography. In the case of GpG we expected that the 3′ G would receive the label, therefore labeled G nucleoside would be released. In the case of poly G we would predict that some liberated nucleotides would be labeled GMP. Autoradiograms in Figure 5 show that this is indeed the case. In Figure 5B it is shown that poly G yields a small amount of labeled, methylated GMP (marked by arrows) as judged by expected migration coordinates of authentic methylated GMP. In the case of labeled GpG, only labeled 1-methyl G nucleoside was detected (Figure 5A). This shows that GpG may fit in the active site in one position relative to 5′ polarity and that GpG is a minimal, albeit inefficient, substrate for the enzyme. This inefficiency is not unexpected since these substrates are far removed from an intact tRNA structure. In both cases cyclic forms were observed as expected following base hydrolysis, yielding two spots.

Methylation of tRNA Substrates with Architectural Perturbations

The above studies establish that GpG is a minimal substrate sequence which must be embedded within a general tRNA structure. If this is true, then the position in space of G^{37} relative to the rest of the molecule should be very important in order that correct tRNA contact sites match enzyme topology in the active complex. For example, if the GpG sequence were repositioned in the anticodon stem−loop structure would the enzyme still be capable of incorporating GpG into the active site for catalysis? To test this idea *in vitro* transcribed variants of $tRNA_1^{Leu}$ were prepared and tested as substrates for 1MGT.

Perturbations in the Anticodon Stem and Loop. One or three extra base pairs were first inserted into the anticodon stem (Figure 6B,C). As can be seen in both cases, adding extra stacked bases led to a marked reduction of catalytic efficiency, which strongly suggests that the relative distance and position of the GpG site must be critically aligned with

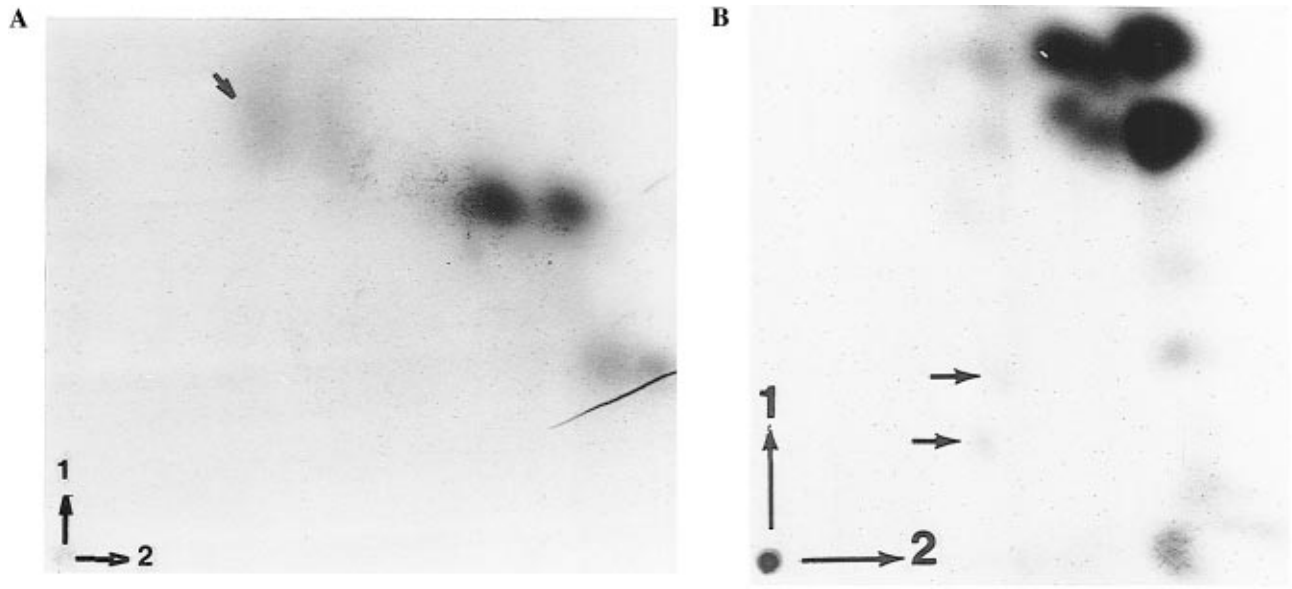

FIGURE 5: Chromatography of labeled nucleotide and nucleoside products from GpG and poly G. (A) Autoradiogram of TLC-separated products released by alkali treatment from GpG. (B) Autoradiogram of TLC-separated products released by alkali treatment from poly G. The numbers "1" and "2" indicate the dimension of separation as outlined in Materials and Methods. In panel A, small arrows indicate the position of methylated G nucleoside labeled with [^{14}C]AdoMet. Two spots indicated are expected cyclic G derivatives obtained as a result of base hydrolysis. Similarly, in panel B are seen cyclic derivatives of GMP methlated and labeled with [^{14}C]AdoMet. In both cases spots co-migrated with authentic methylated G nucleoside or methylated GMP. The unmarked radioactive spots seen are all derivatives of ^{14}C-labeled AdoMet.

the rest of the molecule. The result of these insertions is the spiral downward movement of G^{37} relative to the rest of the tRNA molecule. Substitutions were also made in the anticodon loop which might be expected to disrupt cannonical structure. First, a C then an A was substituted for U at position 32 (Figure 6D,E). The A might be expected to permit base pairing and constrict the size of the anticodon loop, which then should result in marked structural changes. An A substitution but not C resulted in an order of magnitude reduction in V_{max} and an increase in K_m (Table 1). The insertion of three G residues might be predicted to collapse the anticodon loop through base pairing and thereby place the quanine base at position 37 in stacked conformation. In this case (Figure 6F) there was a complete loss of detectable activity, indicating that the important GpG structural motif must be in appropriate conformation for productive interaction with enzyme.

Perturbation in the Amino Acid Acceptor Stem. We next determined if the acceptor stem provided important contacts for the enzyme by testing the methylation capacity of three further variants (Figure 6G,H,I). Three or five base pairs were deleted from the acceptor stem. Alternatively, three base pairs were inserted. It can be seen that extension of the acceptor stem had little effect on activity, but the deletion of three or more base pairs had a remarkable effect on activity (Figure 6). Thus it can be concluded that the acceptor stem structure is required for optimal enzyme activity. In addition, since 1MGT is active with several tRNA's which exhibit little or no sequence homology in the stem (e.g., $tRNA^{His}$ G^{37-36}, $tRNA^{Asp}$ G^{36}) it seems likely that loss of specific base contacts does not explain the poor activities of truncated substrates.

Perturbation in the tRNA Core. Once it was determined that the yeast $tRNA^{Asp}$ *in vitro* transcript was an excellent substrate for 1MGT (Figure 3C), it became possible to utilize the numerous structural variants which have been made and characterized in the past (*19*). For example, mutations in the core structure of $tRNA^{Asp}$ had been prepared and characterized which disrupt tertiary interactions and thus virtually abolish the recognition by cognate tRNA synthetase (*19*). Interestingly, it was possible to prepare compensatory structural mutations which partially restored activity (*19*). Specifically, key tertiary base interactions between $A^{14}-U^{8}$ and $A^{15}-U^{48}$ (Figure 7A) and crucial D loop−T loop interactions (Figure 7C) were disrupted by mutation.

The effect of these structural perturbations and compensatory mutations on methylation by 1MGT was tested by using the same set of variants into which a G residue was additionally inserted at position 36 (Figure 7). Table 1 shows that in the case of $A^{14}-U^{8}$ and $A^{15}-U^{48}$ disruptions (Figure 7A), there is a drastic reduction in V_{max} whereas K_m is not affected. However, compensatory mutations (for cognate tRNA synthetase) in the D loop (Figure 7B) did not restore methylation activity. Interestingly, disruption of the D/T loop interaction (Figure 7C) does not affect V_{max} but increases K_m 4-fold. However, compensatory mutations (for cognate tRNA synthetase; Figure 7D) have no influence on the methylation properties of the tRNA.

DISCUSSION

Initially, it was important to prepare enzyme suitable for the study of tRNA-enzyme recognition, and to determine what role if any the second substrate AdoMet might play in RNA recognition. For example, if binding studies are to be subsequently carried out it would be important that binding studies be done in the absence of methylation.

The mechanism of the reaction as judged by kinetic analysis was found to be random sequential. This is the case for several other RNA methylases (*20*−*22*) and suggests that this may be a common mechanism for this class of enzymes (*22*). In support of this idea, recent results in this laboratory show clearly that enzyme binds tRNA in the absence of AdoMet (Gabryszuk and Holmes, unpublished results).

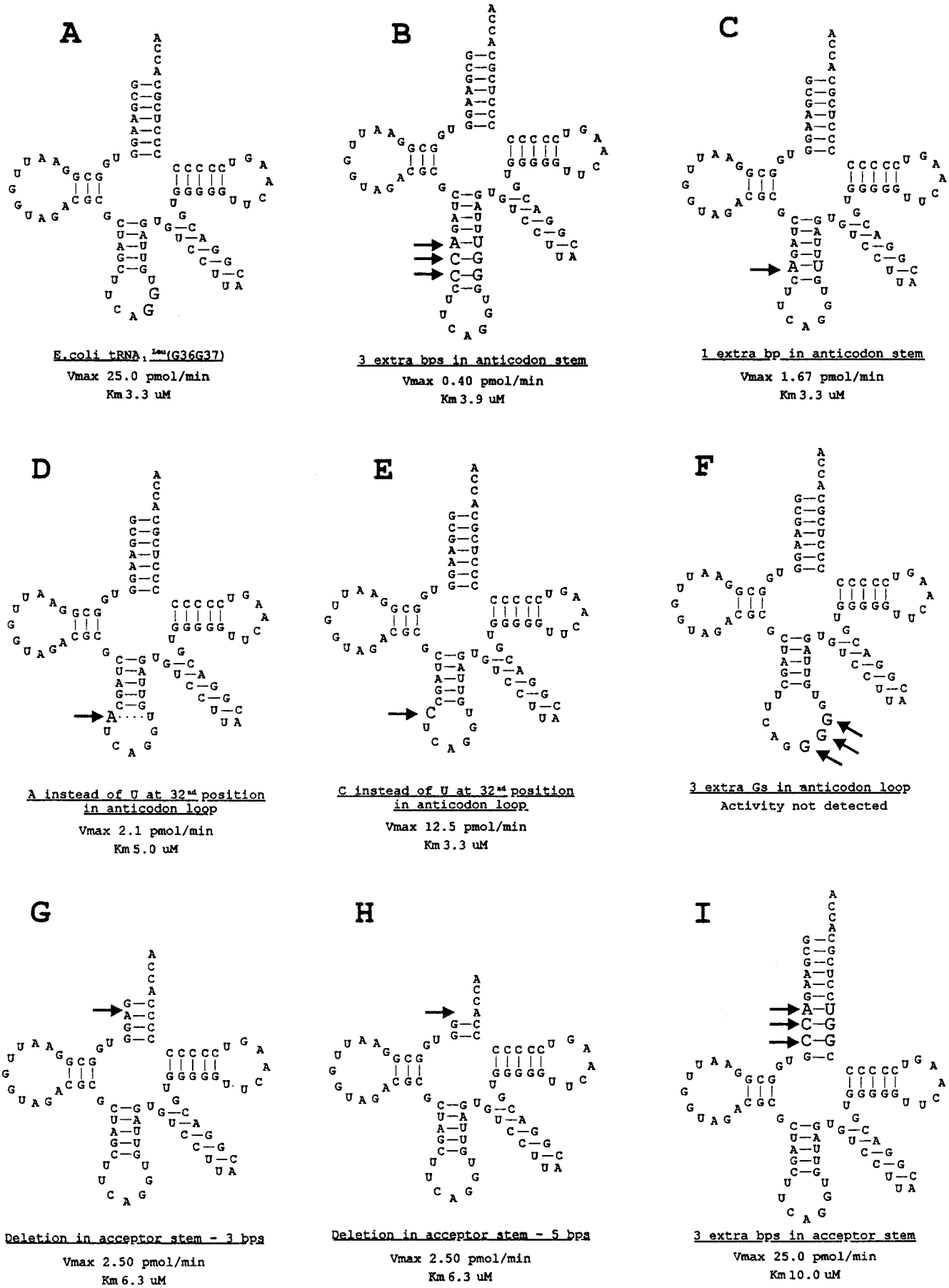

FIGURE 6: Structural variants of *E. coli* $tRNA_1^{Leu}$. (A) Reference $tRNA_1^{Leu}$ transcript. (B, C) Anticodon stem variants. (D−F) Anticodon loop variants. (G−I) Acceptor stem variants. All kinetic values are the averages of three independent determinations done in the presence of excess AdoMet (50 μM). All were assayed in triplicate with 5 μg of pure his-tagged enzyme of equal specific activity. Kinetic values shown are the average of at least three independent assays.

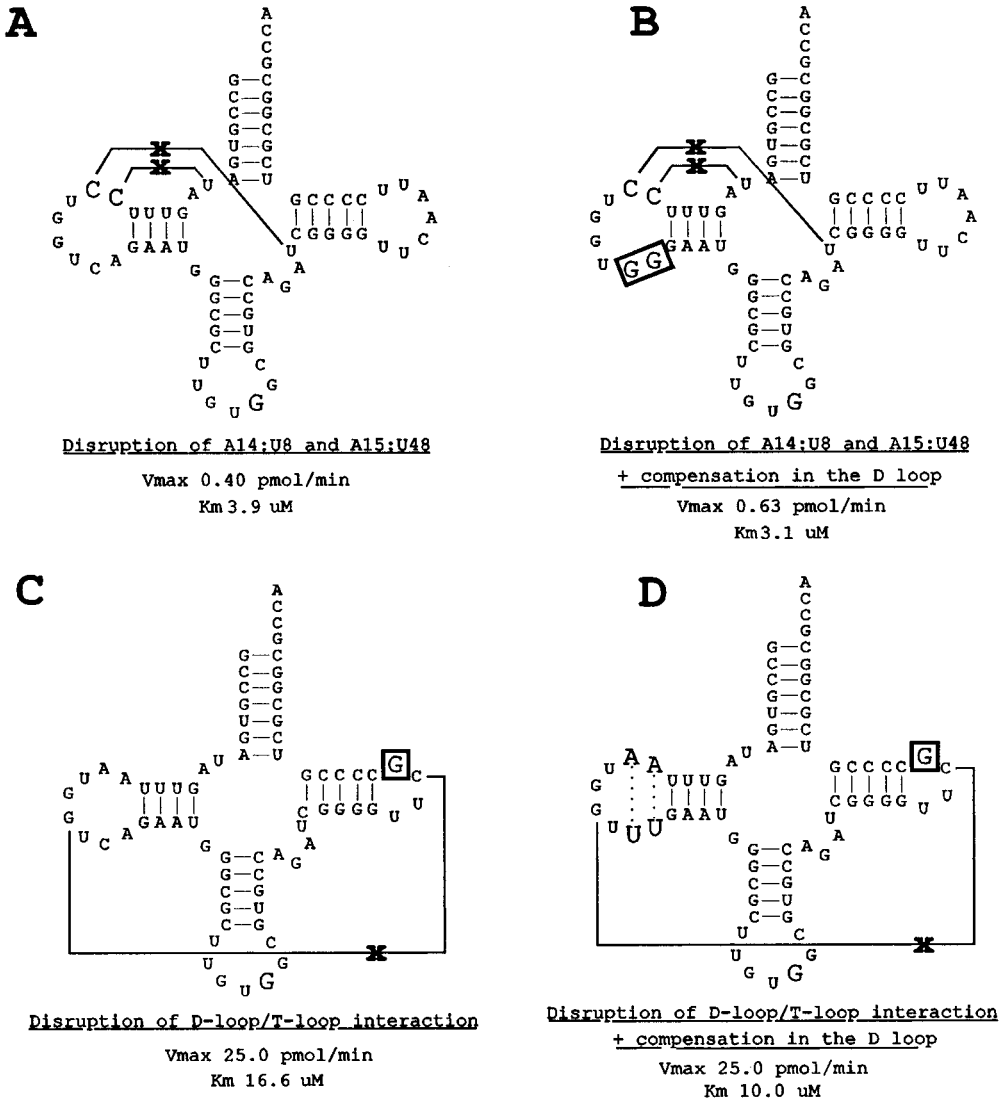

FIGURE 7: Structural variants of yeast $tRNA^{Asp}$ G36,G37. (A, C) Variants which interrupt crucial tertiary interactions. (B, D) Variants with new tertiary interactions which compensate for substitutions in A and C, respectively. All four variants were substituted with G at position 36. The V_{max} values were derived from Lineweaver−Burk plots of at least three independent determinations. All were assayed using 5 μg of pure his-tagged enzyme of constant specific activity which is given in Materials and Methods.

Here we have shown that the dinucleotide sequence $G^{36}pG^{37}$ and the general tertiary structure of tRNA are key determinants for binding and subsequent catalysis. This idea is supported by the finding that several tRNA species with widely divergent primary sequence are substrates for 1MGT, including heterologous *E. coli* $tRNA^{His}$ and *Saccharomyces* $tRNA^{Asp}$. We have also shown that human mitochondrial $tRNA^{Pro}$ is an excellent substrate (H. Brule, M. Holmes, G. Keith, M. Helm, R. Giege, and C. Florentz, manuscript in preparation). In addition, 1MGT can bind several structurally diverse tRNA species with essentially the same affinity even in the absence of critical GpG sequences in the anticodon loop. What is also clear from these competition binding studies is that the G^{37} residue is a key determinant for stabilizing tRNA enzyme complexes because wild type tRNA exhibits a K_m of approximately 3 μM, whereas fully methylated $tRNA_1^{Leu}$ must bind much less avidly since the I_{50} is 62 μM (Table 2). This is expected if methylated product must be released. Moreover, if the general tRNA structure is disrupted, as in the case of the hairpin structure derived from $tRNA_1^{Leu}$, it is clear that binding affinity is drastically reduced in this case and strongly suggests that contacts in other part of the tRNA molecule are important for binding.

Recent work by Grosjean et al. also supports the importance of the tRNA tertiary structure for another m^1G methyltransferase from yeast (*14*, *23*). It is interesting to note that this enzyme apparently does not recognize a GpG sequence since $tRNA^{Asp}$ has a C residue at position 36 rather than a G. For these present studies, we substituted G for C at position 36 so that the *E. coli* enzyme would work efficiently. The wild type $tRNA^{Asp}$ actually gave a V_{max} of of 0.5 pmol/min and a K_m of approximately 10 μM. It will be interesting to know if $C^{36}pG^{37}$ will be a critical dinucleotide required for the eukaryotic enzyme or whether only G^{37} is important for recognition. If so, this could imply that the yeast enzyme might have a fundamentally different binding site. An analysis of the literature indicates that there are several examples in mammalian and archeabacterial tRNA's where this may be the case (*18*).

Other specific nucleotide contacts beside G^{36} and G^{37} might also be important since the V_{max} of $tRNA^{His}G^{37}$ is about 5-fold lower than that of $tRNA_1^{Leu}$. Again, we speculate that this enzyme first binds the general tertiary structure of the

tRNA molecule, however, in order for a stable tRNA−enzyme complex to form, GpG must be present in the correct position in the molecule which then fits in the active site of the enzyme. Once this takes place, catalysis proceeds. Here we have shown that a number of mutations which might be expected to disturb the canonical structure of tRNA in several domains are all quite detrimental. For example, mutations which disrupt tRNA core structure and D/T loop interactions through the elimination of key base stacking and ionic interactions yield poor substrates for 1MGT. Moreover, elimination of the acceptor stem, a hallmark structural domain for tRNA, is quite detrimental.

Dramatic effects are seen when G^{37} is presumably repositioned in space when extra base pairs are added to the helical tRNA anticodon stem. In the case where only one extra stacked base is added to the stem, it can be predicted, on the basis of the well-known helical structure of DNA, that G^{37} should be rotated approximately 30° and be moved about 3 Å down and away from primary tRNA contacts elsewhere in the molecule. This slight, putative change in position is enough to reduce V_{max} at least an order of magnitude. The insertion of more bases (three stacked base pairs) yields even larger effects as might be expected since in this case G^{37} it can be imagined that G^{37} should be rotated about 90° if one assumes that about ten stacked bases in DNA constitute a full turn of the helix. In this case we predict that G^{37} would be on the side of the molecule and repositioned approximately 8 Å from the core structure the molecule. We should note that G^{37} is ordinarily directly below the helical acceptor stem of the molecule and thus is pointing roughly in the same direction as the CCA end of the tRNA. In this connection we see that deletion of stacked base pairs in the acceptor stem has a marked effect on activity and suggests that this part of the molecule is very important. Interestingly, we have previously shown that deleting a base pair from the anticodon stem had little effect on activity and suggests that the enzyme is more flexible in accommodating G^{37} moved in the clockwise direction relative to the helical axis of the anticodon stem. This is consistent with the fact that *E. coli* 1MGT must methylate both type I and II tRNA species. That is, the presence of a large variable loop may not create a steric hindrance problem if the enzyme binds primarily structures away from the variable loop. Previous modeling experiments suggest that the extra loop of a type II tRNA point in roughly the same direction as the T loop (*24*). It will be interesting to determine what common and/or different (if any) structures are bound in type I and II tRNA's. This is an interesting issue since in a previous study we have shown that deletion of the variable loop significantly reduces the catalytic efficiency of a type II tRNA (*12*). If the variable loop is important for binding in type II species, then it will be important to determine which alternate structure(s) are bound in type I tRNA. The simple addition of stacked base pairs to the acceptor stem or extra nucleotides to the CCA terminus has little or no effect on activity and implies that (a) the enzyme makes no crucial contacts with the 3′ end of the molecule and (b) specific base contacts are not important even though the presence of an RNA helical structure is. Overall, the picture clearly emerges that disturbing the position in space of G^{37} or eliminating key phosphate contacts elsewhere in tRNA makes for a poor tRNA substrate. However, since deletions of the acceptor stem do adversely affect activity of transcripts it will be important to determine if enzyme actually makes physical contact with it or if stem deletions simply affect other primary sites for binding in an indirect way by affecting conformation or perhaps even transcript folding.

It appears that local primary and secondary structure of the anticodon loop is very important for maximum catalytic efficiency. Two mutations were made which permit abnormal base pairing between bases in the loop. In one case where three G residues were inserted it appears that the loop might collapse, placing G^{37} in a quasi-double-stranded region. In this case the tRNA was totally inactive and suggests that 1MGT cannot methylate G residues imbedded in helical structure. It will be important to determine just how crucial the fine structure of the AC loop will be for enzyme activity.

We have shown that the dinucleotide GpG is a minimal substrate and must therefore fit into the active site of the enzyme. However, the relative binding affinity is poor (K_i, 6 mM) and demonstrates clearly that optimal methylation requires the tRNA molecule in which it is embedded. Poly G is a somewhat better binding substrate (K_i, 2 mM), which suggests that attached polynucleotides, perhaps of any sequence, may facilitate catalysis via one-dimensional diffusion. It is important to note here that as expected, GpG must enter the active site in a particular orientation because we have demonstrated that only the 3′ G residue is modified, as judged by finding only labeled G after *in vitro* methylation and alkaline digestion. In the case of poly G this was not apparent because presumably enzyme methylated random G residues in the polymer after binding. An interesting question not answered here is, can enzyme methylate a G residue when a methylated base is adjacent to the target G residue? The use of appropriate synthetic dinucleotides might better address this question and better define the fine structural requirements for a good fit in the active site. In addition, appropriate affinity-labeled substrates might be used to identify key amino acid residues in or near the active site(s). The use of poly G is complicated by the fact that much of this polyanion is probably in stacked helical configuration in solution, which might therefore impede methylation. If this were not the case perhaps even better K_m values might have been obtained.

Taken together, the studies with GpG and poly G demonstrate clearly that 1MGT can catalyze the methylation of G in the absence of other tRNA contacts. However, optimal activity is achieved only when GpG is embedded in the general tRNA structure. Consistent with this model are our recent findings that enzyme protects many sites in tRNA outside $G^{36}pG^{37}$ from chemical cleavage (Gabryszuk and Holmes, manuscript in preparation). We have shown that tRNA substrates sharing only the invariant base sequences of tRNA all are good substrates for 1MGT; therefore, we believe that it is likely that many of these key binding sites may involve the sugar-phosphate backbone of the molecule rather than specific base sequences.

The fact that this enzyme can bind and methylate poly G or GpG may reflect the evolutionary origin of this enzyme. The first substrate for this enzyme could have been a simple RNA molecule or even a small oligonucleotide with little or no tertiary structure. Another possibility is that 1MGT may not have been an RNA binding protein initially, but rather a nucleotide binding enzyme requiring nucleotide cofactors like NAD or perhaps an enzyme involved in nucleotide binding or metabolism. This idea may not be

idle speculation since it now appears that many enzymes appear to bind RNA in addition to a catalytic role. In a recent review (*25*) a number of proteins appear to have such a dual function and may have evolved dinucleotide binding pockets which may play a role in RNA binding. For example, the iron regulatory protein (IRP) has recently been shown to be the cytoplasmic enzyme aconitase! Other enzymes such as thymidylate synthase, dihydrofolate reductase, catalase, glyceraldehyde phosphate dehydrogenase (GAPDH), and several other NADP binding proteins all are capable of binding RNA which often are mRNA structures. In this connection, it will be interesting to determine if the *trmD* gene is essential for cell viability since missense mutations studies so far affect only growth rates. If so, this could indicate a second function for the *trmD* polypeptide.

It is interesting to reflect on the origins of the large number of modification enzymes which exist in cells. In the case of bacteria this is probably around 40−50 different proteins, and in the case of eukaryotes, far more, given the complexity of modification in these cells (*9*). Of the modification enzymes sequenced so far there is little obvious sequence homology which might suggest that this family of enzymes may have evolved via convergent evolution of many different genes. We find no remarkable homology in 1MGT with other modification enzymes with the exception of the Ado-Met consensus sequence noted in other methyltransferases.

This lack of homology is consistent with the finding that RNA structures recognized by a variety of modification enzymes are highly variable. For example, in the case of the enzyme that methylates G26 into $m^2{}_2G$ in many eukaryotic tRNA species, the complete structure of tRNA is required (*13*, *14*, *23*). Two other enzymes previously thought to recognize mainly simple nucleotide determinants [the enzyme that transforms the adenosine at position 34 to inosine (*26*) and the Q34 insertase that exchanges guanine for queuosine (*27*)] now have been shown to require correct 3D structure (*14*, *28*). Certain other enzymes do not require an intact tRNA structure: such is the case with m5U54 methyltransferase that only requires primary and secondary structures of the T arm (*29*) and with the enzyme that methylates the conserved adenosine residue at position 58 into m1A58 (*28*). This enzyme can transfer the methyl group to the 3′ half of the tRNA. Yet another group of enzymes does not rely on nearby sequences such as the enzyme that convert G^{37} into m^1G^{37} in eukaryotic tRNA's (*14*). In light of these observations no simple scheme can be formulated for tRNA recognition for modification, and, like tRNA synthetases, there will be several classes of enzymes with distinct requirements for recognition.

Ultimately, detailed NMR and X-RNA structural information concerning tRNA recognition by a spectrum of modification enzymes will be required if we are to fully understand how RNA structural determinants are bound and modified. It will be interesting to determine if 1MGT may have multiple functions, as has been shown to be the case with the trmA gene product (*9*). We currently are carrying out experiments aimed toward these ends.

ACKNOWLEDGMENT

We particularly thank Drs. Bernard and Chantal Erhesmann, Joe Ng, Joele Rudinger, Anne Theobald, and many others at the I.B.M.C., including Mark Helm, Danielle Werling, Herve Brule, Dr. Bernard Lober, and Marie Sissler for their great interest and support for this project and for a great experience at the institute while one of us (W.M.H.) was in Strasbourg on sabbatical leave in 1995.

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BI9701538