Transfer RNA Contains Sites of Localized Positive Charge: Carbon NMR Studies of [^{13}C]Methyl-Enriched *Escherichia coli* and Yeast tRNA^{Phe}†

Paul F. Agris,*,‡,§ Hanna Sierzputowska-Gracz,‡ and Christine Smith‡
Division of Biological Sciences and Department of Medicine, University of Missouri, Columbia, Missouri 65211
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ABSTRACT: The possibility of positively charged nucleosides in tRNA has been suspected because certain posttranscriptional methylations produce quaternary nitrogens. To investigate this possibility and the importance of such methylations to tRNA structure, we have continued our studies of [^{13}C]methyl-enriched phenylalanine tRNA of *Escherichia coli* [Kopper, R. A., Schmidt, P. G., & Agris, P. F. (1983) *Biochemistry 22*, 1307-1401] and yeast [Smith, C., Petsch, J., Schmidt, P. G., & Agris, P. F. (1985) *Biochemistry 24*, 1434-1440]. *E. coli* and yeast tRNA were ^{13}C-enriched in their methyl groups in vivo, and phenylalanine-specific tRNA was isolated. Methyl proton and carbon signal assignments were confirmed and correlated for the purified tRNAs under native conditions via the first application of two-dimensional carbon-proton correlation NMR spectroscopy to a native nucleic acid. The methyl proton chemical shift of the 7-methylguanosine (m^{7}G) signal from tRNA was easily determined, although by conventional ^{1}H NMR spectroscopy it would have been hidden by ribose resonances and H_2O. The chemical shift for 1-methyladenosine (m^{1}A) protons was shown to be 3.01 ppm. Resolution of close or overlapping peaks was greatly enhanced by the two-dimensional experiment especially for the proton methyl resonances. In addition, proton-carbon chemical shift correspondence has been determined for the two 5-methylcytidines (m^{5}C's), the methyl esters of wybutosine (Y), and the two ribose methyl groups, Gm and Cm, of yeast tRNA^{Phe}. Thermal denaturation and Mg^{2+} depletion affect the methyl carbon NMR chemical shifts of tRNA. Methyl resonances of m^{7}G and m^{1}A moved upfield in a direction opposite to all the other resonances of ^{13}C-enriched tRNA. In order to understand these changes in chemical shifts, solution conditions of the mononucleosides were varied in an attempt to mimic the microenvironment of m^{7}G and m^{1}A in tRNA. The carbon chemical shifts of the methyls of m^{1}A and m^{7}G in native tRNA were reproduced by solutions of the fully protonated mononucleosides. The methyl group carbon chemical shifts suggest that the nucleosides are fully protonated in the native, three-dimensional structure of tRNA. The donated proton from base pairing implements the full positive charge on the base ring. We propose that the positive charge and not the atomic structure of the methyl group may be a major effector of local secondary and tertiary interactions, as seen in the X-ray crystallographic structure of yeast tRNA^{Phe}. The positive charge may also be important for common protein recognition of the many tRNAs with m^{7}G and/or m^{1}A.

We have been utilizing site-specific ^{13}C enrichment of tRNA to study its native structure, internal motion, and function (Schmidt et al., 1980, 1983; Kopper et al., 1983). Carbon-13 enrichment of the naturally occurring methyl groups in tRNA has been accomplished in vivo by selection of *Escherichia coli* and yeast mutants and growth conditions that result in the efficient incorporation of the methyl group from [*methyl*-^{13}C]methionine (Tompson & Agris, 1979; Agris et al., 1983). Enrichment is better than 70 atom % and almost exclusively in the methyl groups (Agris et al., 1983). Carbon NMR spectra of purified *E. coli* tRNA^{Phe} exhibited three prominent, well-resolved methyl signals easily assigned to its three methylated nucleosides: ribosylthymine (T, m^{5}U), 2-(methylthio)-N^6-(Δ^2-isopentenyl)adenosine (ms^{2}i^{6}A), and 7-methylguanosine (m^{7}G) (Kopper et al., 1983; Agris & Schmidt, 1980). Carbon NMR spectra of purified yeast tRNA^{Phe} exhibited 12 resolved methyl signals, somewhat more difficult to assign to 13 of the 14 naturally occurring methyls in the tRNA: T; two 5-methylcytidines (m^{5}C); N^2-methylguanosine (m^{2}G); 1-methyladenosine (m^{1}A); the two methyls of N^2,N^2-dimethylguanosine (m$^2_2$G); m^{7}G; *N*-methyl of wybutosine (Y); two methyl esters of Y; the two ribose methyls of Gm and Cm (Smith et al., 1985). A thirteenth ^{13}C-enriched resonance recently has been assigned to carbon 10 of Y (H. Sierzputowska-Gracz, unpublished results).

Fifty percent of all tRNAs contain m^{7}G in their extra loops, and some 20% of eucaryotic tRNAs contain m^{1}A in their T-loops (Agris & Kopper, 1983). Both methylations produce quaternary nitrogens. Thus, it had been suspected that these two nucleosides are the major contributors of positive charges to tRNA molecules under physiological conditions. The two nucleosides are involved in tertiary structure hydrogen bonding as determined from the X-ray crystallographic structure of yeast tRNA^{Phe} (Sussman et al., 1978; Holbrook et al., 1978) and the proton nuclear magnetic resonance (NMR) spectroscopy of a number of tRNAs (Hare & Reid, 1982; Roy et al., 1982; Roy & Redfield, 1983). Here, we confirm the proton and carbon signal assignments for these two nucleosides, correlate all methyl ^{1}H and ^{13}C signals under native structural conditions, and report the first two-dimensional heteronuclear ^{13}C-^{1}H correlation spectroscopy of any biologically functional nucleic acid. In order to determine whether or not m^{7}G and m^{1}A carry charges within the native structures of the *E. coli* and yeast tRNA^{Phe}, and if so what are the charge distributions,

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‡ Division of Biological Sciences.
§ Department of Medicine.

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it was necessary to compare NMR spectra of the tRNAs with those of the mononucleosides under the same physiological conditions.

Materials and Methods

Preparation of [^{13}C]Methyl-Enriched Yeast and E. coli tRNA^{Phe}. *Saccharomyces cerevisae* met$^-$trp$^-$ (RH 762) was grown at 30 °C in minimal medium supplemented with L-[*methyl*-^{13}C]methionine and L-tryptophan, as previously described (Agris et al., 1983). *E. coli* C6 rel$^-$met$^-$cys$^-$ strain M1 was grown according to our published method (Tompson & Agris, 1979). Cells were harvested, and their tRNA was extracted according to published methods (Agris et al., 1983). Transfer RNA^{Phe} was purified from the unfractionated *E. coli* or yeast tRNA by benzoylated diethylaminoethylcellulose and reversed-phase (RPC-5) chromatography. Purity of the two tRNA^{Phe} was determined to be greater than 85% by (a) comparison of aminoacylations with phenylalanine, leucine, serine, tyrosine, and tryptophan, (b) urea–polyacrylamide gel electrophoresis, and (c) quantitative high-performance liquid chromatography (HPLC) analysis of nucleosides resulting from a nuclease P1 and bacterial alkaline phosphatase digestion of a small aliquot (Davis et al., 1979; Gehrke et al., 1983). The tRNAs were prepared for NMR by extensive dialysis against ethylenediaminetetraacetic acid (EDTA) in glass-distilled water and then against glass-distilled water. Samples were concentrated by vacuum, exchanged twice with D_2O, and placed in phosphate-buffered saline (PBS) of pH 7.2 in D_2O (10 mM $MgCl_2$, 15 mM KH_2PO_4, 10 mM Na_2HPO_4, 100 mM NaCl). Final concentrations were approximately 1.3 mM tRNA in a volume of 0.6 mL.

NMR Spectroscopy. Spectra were obtained with a Nicolet 300-MHz (^1H) Fourier-transform spectrometer with an Oxford Instruments wide-bore magnet. The instrument was equipped with a Nicolet 5-mm single-frequency (75.5 MHz) ^{13}C probe and a Doty Scientific single-frequency ^{13}C preamplifier.

Transfer RNA methyl carbon signal assignments were accomplished as previously published (Agris et al., 1983; Tompson et al., 1979). Proton and carbon resonances were correlated by the two-dimensional heteronuclear (carbon–proton), absolute magnitude correlation spectroscopy reported here. Two-dimensional spectra were collected as 64 blocks of 2048 data points from 1100 free induction decays for each block. A total cycle time of 3.5 s was used; sweep width was ±2326 Hz in the ^{13}C dimension, and quadrature phase detection was used to observe the methyl region alone. The carbon carrier frequency was located at the center of the methyl region at 36 ppm. A proton frequency range of 1290 Hz (0–4.3 ppm) was covered. In the carbon dimension, the digital resolution was 4.6 Hz, whereas in the proton dimension it was 20 Hz. The proton sweep width was shortened to 1020 Hz (0–3.4 ppm) for the *E. coli* spectrum to enhance resolution since only the one methyl group signal of m^{7}G was downfield of that for T and ms^{2}i^{6}A. Thus, the methyl resonance for m^{7}G appears in the plot (Figure 1B) as being folded-in. The "proton spectrum" at top of the contour plot is a summation of all the 90° projections on the ^{13}C spectrum.

Results

Carbon–proton two-dimensional chemical shift correlation spectra were taken of ^{13}C-enriched yeast and *E. coli* tRNA^{Phe} under native conditions in PBS at 25 °C. Contour plots of the methyl regions are shown in Figure 1. A ^{13}C spectrum is on the left of the figures. The problem of assigning methyl protons to signals close to and under the C–OH/H_2O region is dramatically illustrated by the true proton spectrum without carbon decoupling at the top of the yeast tRNA contour plot (Figure 1A).

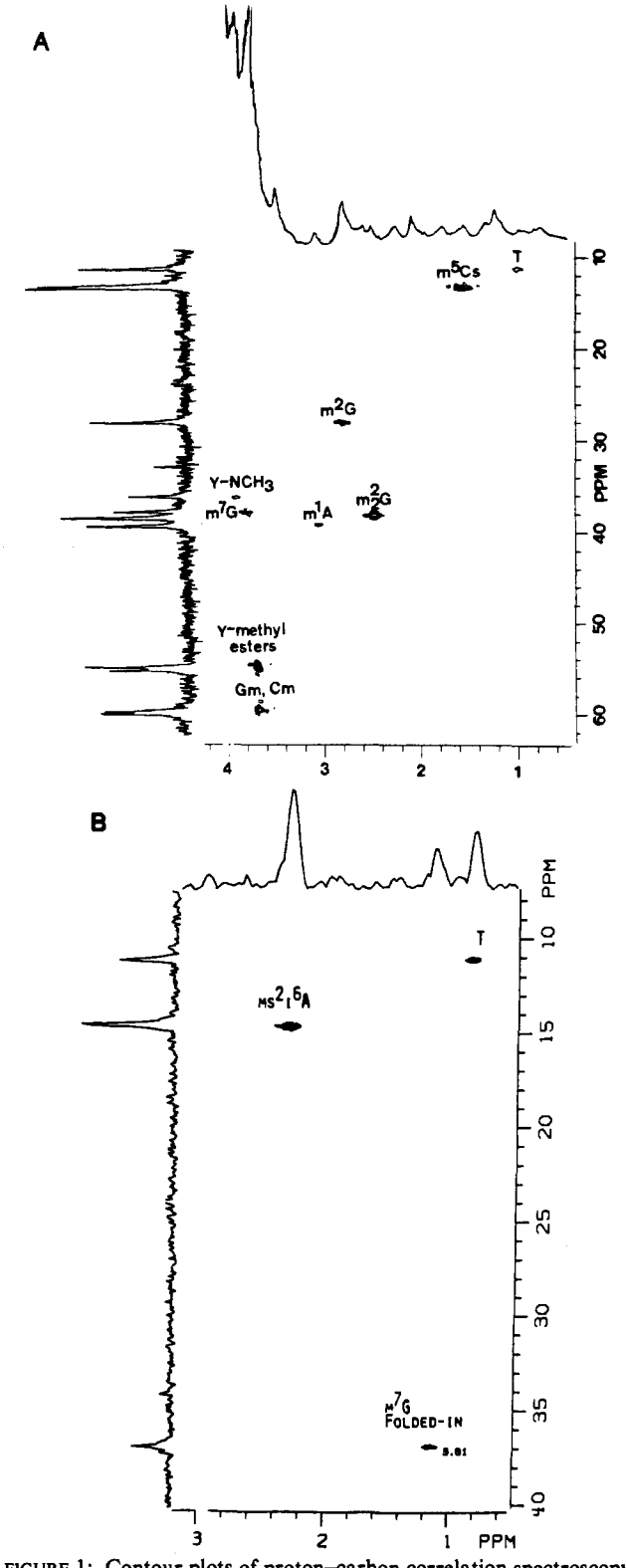

FIGURE 1: Contour plots of proton–carbon correlation spectroscopy. Two-dimensional spectroscopy was accomplished with [^{13}C]-methyl-enriched yeast (A) and *E. coli* (B) tRNA^{Phe} molecules as described in the text. A ^{13}C NMR spectrum is on the left of both plots. A carbon-coupled, proton spectrum is shown at the top of the figure for yeast tRNA^{Phe} (A), whereas the summation of all projections (90°) on the carbon spectrum is shown at the top of the plot for *E. coli* tRNA^{Phe} (B). The signal for m^{7}G in the *E. coli* spectrum is folded in due to a shortened ^1H sweep width.

Table I: Carbon–Proton Chemical Shift Correlations for ^{13}C-Enriched Methyls of Yeast and *E. coli* tRNA^{Phe}

nucleoside identity and sequence position	chemical shift[a] proton yeast	proton *E. coli*	carbon yeast	carbon *E. coli*
T-54	1.00	0.85	11.0	11.14
m^5C-(40 or 49)	1.52		12.9	
m^5C-(40 or 49)	1.57		12.7	
$ms^2(i^6A)$		2.31		14.70
m_2^2G-26	2.44		37.6	
m^2G-10	2.76		27.4	
m^1A-58	3.01		38.4	
Gm-34	3.59		58.7	
Cm-32	3.66		58.4	
Y methyl ester-37	3.66		53.9	
Y methyl ester-37	3.67		53.6	
m^7G-46	3.76	3.81	36.9	37.12
N-methyl-Y-37	3.87		35.4	

[a] Chemical shifts (ppm) are relative to dioxane at 3.53 ppm for proton and 67.4 ppm for carbon. Signal correlations are made from carbon-observe two-dimensional heteronuclear, carbon–proton correlation spectroscopy, except for that of the Y methyl esters accomplished with proton-observe, carbon-detect spectroscopy.

Table II: Methyl Carbon Chemical Shifts[a] for Mononucleosides and tRNA

ionic strength [Na (mM)]	m^1A	m^7G	temp (°C)
1	36.7	36.6	22
5	36.7	36.6	
10	36.9	36.9	
100	37.3	36.6	
500	37.1	36.6	
1000	37.1	36.7	
hydroiodide[b]	38.7	36.9	22
tRNA[c]	38.4	36.9	22
	37.0	36.3	40
	36.3	36.3	60

[a] Carbon chemical shifts (ppm) are relative to dioxane at 67.4 ppm. [b] Values for the hydroiodide salts were first reported by Box et al. (1981) and confirmed by us under physiological conditions. The chemical shift for m^7G-HI (36.9 ppm) was found identical with that for m^7G plus equimolar TFA in otherwise analogous solution conditions. [c] Values for tRNA from Tompson et al. (1979) and Agris et al. (1983).

Carbon–proton signal correlations from the 90° projections on the carbon spectra and their assignments are shown in Table I for both *E. coli* and yeast tRNA^{Phe} methyl groups. Four sets of carbon peaks displayed closely spaced lines in the ^{13}C NMR spectrum. The two methyls of m_2^2G-26 showed chemical shift equivalence at 37.6 ppm. The two m^5C's (12.7 and 12.9 ppm) at nucleoside positions 40 and 49, the two wybutosine (Y) methyl esters (53.6 and 53.9 ppm) at position 37, and the two ribose methyls (58.4 and 58.7 ppm), Gm-34 and Cm-32, presented resolved signals. Two-dimensional chemical shift correlation spectroscopy yielded high enough resolution for us to correlate the proton with the carbon signals for these sets of resolved methyls. The upfield carbon signal at 12.7 ppm for one of the m^5C's was associated with the downfield proton signal at 1.56 ppm. The reverse is true for the other m^5C. These signals have not as yet been unquestionably assigned to their particular nucleosides, m^5C-40 or m^5C-49 (Heershap et al., 1983). They could be differentiated in the future from nuclear Overhauser measurements (Johnston & Redfield, 1978; Hare & Reid, 1982; Heershap et al., 1983) or via experiments employing stepwise melting (Boyle et al., 1983) of the ^{13}C-enriched tRNA structure since the two nucleosides are located in different stem regions. The Y methyl ester proton peaks were too close to differentiate with certainty given the digital resolution used here. However, we were able to differentiate the two Y methyl ester proton signals and correlate them to their respective carbons using the complementary two-dimensional technique of proton-observe and carbon-detect spectroscopy with a select narrow spectral range of less than 1 ppm.

The methyl carbon resonance at 58.4 ppm has been assigned to Cm, and the signal just downfield of it at 58.7 ppm has been assigned to Gm (Kopper et al., 1983; Agris et al., 1983). We can add information to the proton signal assignments under native conditions by demonstrating that the upfield Cm carbon signal correlated with a downfield proton signal. Gm methyl protons appeared to split into two peaks, one coincident with Cm and the other upfield of the Cm signal. The Gm proton peaks may be reporting two conformations of the anticodon loop under native conditions (Sierzputowska-Gracz, personal communication).

The two-dimensional heteronuclear spectra taken of *E. coli* and yeast tRNA^{Phe}s under identical conditions have demonstrated differences in ^{13}C and ^{1}H chemical shifts for the two methyls common to both molecules (Table I). These differences were verified by one-dimensional ^{1}H and ^{13}C spectra. The most common of methylated ribonucleosides, T-54, was found to differ between the two tRNA^{Phe} in methyl carbon chemical shift by 10.5 Hz (0.14 ppm), the equivalent of a single line width, and in proton by 45 Hz (0.15 ppm). The second most common methylated nucleoside in tRNA, m^7G-46, displayed methyl resonances that differed by 16.5 Hz (0.22 ppm) in carbon and by 15 Hz in proton. All methyl proton peaks previously assigned by other laboratories (Davanloo et al., 1979) were confirmed with one exception. A resonance at 3.01 ppm attributed to dihydrouridine-16 actually arises from m^1A.

In carbon NMR spectra, methyl resonances of m^7G and m^1A in *E. coli* and yeast tRNAs changed chemical shifts in directions opposite to all other carbons in response to structural changes. Contrary to the general trend, methyl signals of m^1A and m^7G moved upfield upon heat denaturation of the tRNA (Table II) and downfield with addition of Mg^{2+} to extensively dialyzed tRNA solutions (Tompson et al., 1979; Agris & Schmidt, 1980; Kopper et al., 1983; Smith et al., 1985). Therefore, the contributions made by m^7G and m^1A to the native structure, stability, and function of tRNA may be different from all other nucleosides and may be considerable. The special character of the two nucleosides as evidenced by the contrary chemical shift changes may reside in their having quaternary nitrogens, being protonated, and, thus, being positively charged in tRNA.

The methyl carbon chemical shift of the mononucleoside m^1A was found to be 37.3 ppm in PBS, 100 mM Na^+, pH 7.2 (Table II), whereas in tRNA the methyl resonance of m^1A was 38.4 ppm. Increasing ionic strength affected the chemical shift of the mononucleoside's methyl resonance by moving it downfield toward that of its signal in tRNA. The methyl carbon resonance of the mononucleoside m^7G was upfield (36.6 ppm) of that in tRNA (36.9 ppm) and was not as affected by ionic strength but corresponded exactly to that in tRNA with addition of equimolar trifluoroacetic acid (TFA) to solutions of the mononucleosides. *The methyl carbon chemical shifts of the hydroiodide salts of both nucleosides compared very favorably to that of the nucleosides in tRNA under native conditions at 22 °C (Table II).* Reducing the pH of the PBS solutions of the two mononucleosides (pH 7.0–5.4) had a slight effect on the m^1A methyl carbon signal chemical shift. It moved away from that in tRNA (Δ = 0.4 ppm upfield at low

pH). No effect was evident for the methyl of m^7G. Experiments at higher pH were avoided due to the lability of the nucleosides.

To confirm that the correspondence of tRNA methyl carbon chemical shifts to that of m^1A·HI and m^7G plus TFA was truly attributable to the protonated forms of the mononucleosides, we have characterized the neutral and protonated mononucleosides by ^1H, ^{13}C, and ^{15}N NMR spectroscopy under physiological conditions comparable to that for tRNA and by IR spectroscopy. This investigation (H. Sierzputowska-Gracz and P. F. Agris, unpublished results) also revealed the sites of protonation and utilized model compounds, ethenoadenosine (Sierzputowska-Gracz et al., 1984) and ethenocytidine (Jaskolski et al., 1981; Kozerski et al., 1984). The hydroiodide of m^1A corresponded to the protonated form of the nucleoside. The spectral changes resulting from addition of equimolar TFA to solutions of m^7G also were attributable to the protonated form of this nucleoside, whereas the neutral form (zwitterion) was present before addition of acid.

Discussion

Methyl carbon resonances enriched in vivo provide easily identifiable signals under native conditions (Kopper et al., 1983). The culturing of selected *E. coli* (Schmidt et al., 1980; Tompson & Agris, 1979) or yeast (Agris et al., 1983) strains with ^{13}C-enriched precursors of nucleic acids and the blocking of de novo purine synthesis by using either mutants and/or drugs ensures the incorporation to greater than 70 atom % ^{13}C at known locations. This has allowed us to perform the first two-dimensional heteronuclear carbon–proton chemical shift correlation Fourier-transform NMR spectroscopy on two native nucleic acids, the *E. coli* and yeast $tRNA^{Phe}$ molecules. A proton-observe, nitrogen-detected, two-dimensional multiple quantum method has been used for NMR investigations of imino protons in ^{15}N-enriched yeast $tRNA^{Phe}$ (Griffey et al., 1983; Roy et al., 1984) and *E. coli* $tRNA^{Phe}$, $tRNA^{Met}$, and $tRNA^{Lys}$ (Davis et al., 1986). We applied this method to [^{13}C]methyl-enriched yeast $tRNA^{Phe}$ by observing the proton signals with carbon sensitivity. The results successfully complemented that of the two-dimensional carbon-observed investigation. However, attempts at correlation experiments with distortionless enhancement by polarization transfer (DEPT) (Pegg & Bendall, 1983) were unsuccessful for the tRNA macromolecule. Possibly, the enhancement capabilities of DEPT, which are evident for small molecules (Rutar & Wong, 1983), are negated because the T_1 relaxation times for the methyl protons (3 s) are longer than those for the methyl carbons (1.5 s) in the tRNA.

Carbon-13 resonances from methyl groups are well resolved in ^{13}C NMR spectra of tRNA (Kopper et al., 1983; Agris et al., 1983). Some of the methyl proton resonances of tRNA are likewise well resolved in ^1H NMR spectra (Kastrup & Schmidt, 1975, 1978), but definitely the methyl carbon resonances are more easily assigned (Kopper et al., 1983), particularly under conditions where the molecule is in its native state. Some methyl proton signals of yeast $tRNA^{Phe}$, Y NCH_3, m^7G, Gm, Cm, and Y esters, are not easily observed in one-dimensional proton NMR spectroscopy under native conditions because they lie close together on the shoulder of the dominant ribose proton region. Two-dimensional experiments under the same conditions dramatically increased resolution of these groups and eliminated interference from ribose resonances (Figure 1).

Accurate and complete signal identification of tRNA under native conditions was necessary to embark upon a detailed investigation of m^1A and m^7G function in tRNA. To first establish the presence or absence of charge in these nucleosides in native $tRNA^{Phe}$, we sought solution conditions under which the methyls of the mononucleosides, as model compounds, displayed carbon chemical shifts and chemical shift changes with conditions equivalent to those for the nucleosides in the tRNAs. The carbon chemical shifts of the methyl carbons of the neutral m^1A and zwitterionic m^7G nucleosides in PBS were found to be upfield of those in the tRNAs (Table II). Interpolating the data from Table II shows that improbably high ionic strengths would be required to move the m^1A signal to match that of the one found in the tRNA molecule. Hydroiodide salts of m^1A and m^7G had ^{13}C chemical shifts essentially identical with chemical shifts of m^1A and m^7G in tRNA. On the basis of the corresponding proton chemical shifts in $tRNA^{Phe}$, ring currents would contribute only a fraction to the observed carbon chemical shifts. Thus, the ^{13}C NMR studies showed that these two nucleosides in tRNA under conditions favoring the native tertiary structure of the nucleic acid had electronic configurations similar to that of halide salts of the mononucleosides m^1A and m^7G in PBS.

The ^1H, ^{13}C, and ^{15}N NMR data and IR spectra for the halide salts of m^1A and m^7G, and m^7G plus TFA, were all completely consistent with the respective, fully protonated structures (H. Sierzputowska-Gracz and P. F. Agris, unpublished results). Nitrogen-15 NMR chemical shift is considered one of the best spectroscopic criterium for assessing protonation of the purine ring of nucleosides (Sierzputowska-Gracz et al., 1984). The protonated nitrogen has a very large chemical shift 30–45 ppm upfield from the corresponding unprotonated atom. However, we had found that the ^{13}C chemical shift is completely consistent with ^{15}N data and also is a very useful parameter for analysis of the purine ring protonation.

Functions of m^1A and m^7G in tRNA. The mononucleosides m^1A and m^7G have p*K*'s around pH 7–7.5. We would expect that the mononucleosides are approximately 50% protonated in PBS, pH 7.2, at 22 °C. However, full protonation was reflected in the downfield methyl carbon chemical shifts of the nucleosides in tRNA being analogous to that of the fully protonated mononucleosides. We propose that full protonation in tRNA is achieved through tertiary structure base pairing. The nucleosides in thermal-denatured or Mg^{2+}-depleted tRNA lack the tertiary structure base pairing and are exposed to solvent and, thus, have approximately 50% protonation, which is seen in their upfield methyl carbon chemical shift. Thus, tertiary structure base pairing and the appropriate methylation ensures the existence of strong positive charges at specific sites in the tRNA structure.

The keto–enol, and enamine–ketamine, tautomerism of DNA and RNA major nucleosides has long been shown important to molecular genetics for specifying base pairs. Protonation plays an important role in determining the base form available for pairing. In the case of m^1A studied here, methylation has also played an important role by changing the base pairing, and protonation site possibilities, viz., blocking the N-1 position. Once H bonding has occurred from m^1A-58 to T-54 by a reversed Hoogsteen base pair, the protonated six-membered ring of m^1A is positively charged in yeast $tRNA^{Phe}$, whereas in *E. coli* $tRNA^{Phe}$, for which no crystal structure has been determined, a similar base pairing could be made to unmodified A-58 without production of positive charge. The two tRNAs have T-54 and A-58 in the same nucleotide sequence, GTΨCGAU. Methylation of G-46 to m^7G-46 produces a zwitterion with a positive charge in the imidazole ring. The m^7G positive charge is localized by base pairing through elimination of the zwitterion negative charge

on the pyrimidine ring. In the yeast $tRNA^{Phe}$ structure, m^7G-46 is known to be in a non-Watson–Crick base pair with G-22, which is hydrogen bound to C-13; in the *E. coli* structure, the exact same bonding is possible. However, the nucleotide sequence in which m^7G is found in yeast $tRNA^{Phe}$, AGm^7GU, is quite different from that of *E. coli*, GUm^7GX, where X is 3-(3-amino-3-carboxypropyl)uridine.

Methylation occurs at the time of posttranscriptional processing of the precursor tRNA molecules (pre-tRNA) in *E. coli* and yeast. Prior to methylations at G-46 in the *E. coli* and yeast pre-$tRNA^{Phe}$ molecules, and at A-58 in the yeast pre-$tRNA^{Phe}$ molecule, no positive charges would exist, even in the presence of tertiary structure. There is evidence that undermodified precursors do take a tertiary structure similar to that of mature tRNA. Some modifications are complete before sizing and splicing. However, the final result for both $tRNA^{Phe}$s is a mature tertiary structure with site-specific positive charges. A methyl group is small in atomic size in comparison to the large change in electronic structure implemented by the simple synthesis of m^7G and m^1A in tRNA. Biosynthesis of some modified nucleosides with additional heteroaromatic rings can be extremely complex. Production of m^1A and m^7G by methylation is possibly the smallest alteration of a nucleoside in tRNA to produce a large change in nucleoside character, other than formation of dihydrouridine. In contrast, the methyls of m^2G, m_2^2G, T, and m^5C, for example, impart a small increase in hydrophobic nature to their respective major nucleosides, as readily evidenced by comparing retention times in hydrophobic column chromatography (Gehrke et al., 1983). The methyls of m^7G and m^1A in yeast $tRNA^{Phe}$ are positioned toward the outside of the molecule in the X-ray crystallographic structure. *Therefore, we conclude that the purpose of the methylations at A-58 and G-46 is primarily for production of site-specific positive charges and not for additions of the methyl groups to the tRNA's molecular structure.*

The functions of these positive charges, secured by tertiary H bonding, could be for molding the tRNA tertiary structure, or for protein recognition, or both. Transfer RNAs interact with many proteins, including aminoacyl-tRNA synthetases, elongation and initiation factors, and ribosomal proteins. The positively charged imidazole and pyrimidine rings of m^7G and m^1A, respectively, are on the outside of the molecular structure toward solvent, and thus accessible to interacting proteins. Slight flexibility in the tertiary structure, such as breakage of H bonds at m^1A or m^7G, would result in a considerable reduction of the site-specific positive charges. As early as 1976, Leonard and co-workers (Doree et al., 1976) noted the importance of the positive charge on the base 1-methyladenine for recognition of this hormone by starfish oocyte m^1Ade membrane receptors. If the charges are sites of protein binding, then the commonality of m^7G and m^1A in tRNAs speaks for a common protein recognition, as well as a common recognition mechanism.

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Registry No. rT, 1463-10-1; m^7G, 20244-86-4; m^1A, 15763-06-1; ms^2i^6A, 20859-00-1; Y, 55196-46-8; Cm, 65-46-3; Gm, 118-00-3.

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NMR Studies of Conformations and Interactions of Substrates and Ribonucleotide Templates Bound to the Large Fragment of DNA Polymerase I†

Lance J. Ferrin and Albert S. Mildvan*
Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205
Received December 26, 1985; Revised Manuscript Received March 20, 1986

ABSTRACT: The large fragment of DNA polymerase I (Pol I) effectively uses oligoribouridylates and oligoriboadenylates as templates, with kinetic properties similar to those of poly(U) and poly(A), respectively, and has little or no activity in degrading them. In the presence of such oligoribonucleotide templates, nuclear Overhauser effects (NOE's) were used to determine interproton distances within and conformations of substrates bound to the large fragment of Pol I, as well as conformations and interactions of the enzyme-bound templates. In the enzyme–oligo(rU)$_{54\pm11}$–Mg^{2+}dATP complex, the substrate dATP has a high anti-glycosidic torsional angle ($\chi = 62 \pm 10°$) and an O1′-endo/C3′-endo sugar pucker ($\delta = 90 \pm 10°$) differing only slightly from those previously found for enzyme-bound dATP in the absence of template [Ferrin, L. J., & Mildvan, A. S. (1985) *Biochemistry 24*, 4680–4694]. Both conformations are similar to those of deoxynucleotidyl units of B DNA but differ greatly from those of A or Z DNA. The conformation of the enzyme-bound substrate analogue AMPCPP ($\chi = 50 \pm 10°$, $\delta = 90 \pm 10°$) is very similar to that of enzyme-bound dATP and is unaltered by the binding of the template oligo(rU)$_{54\pm11}$ or by the subsequent binding of the primer $(Ap)_9A$. In the enzyme–oligo(rA)$_{50}$–Mg^{2+}TTP complex, the substrate TTP has an anti-glycosidic torsional angle ($\chi = 40 \pm 10°$) and an O1′-endo sugar pucker ($\delta = 100 \pm 10°$), indistinguishable from those found in the absence of template and compatible with those of B DNA but not with those of A or Z DNA. In the absence of templates, the interproton distances on enzyme-bound dGTP cannot be fit by a single conformation but require a 40% contribution from a syn structure ($\chi = 222°$) and a 60% contribution from one or more anti structures. The presence of the template oligo(rU)$_{43\pm9}$ simplifies the conformation of enzyme-bound dGTP to a single structure with an *anti*-glycosyl angle ($\chi = 32 \pm 10°$) and an O1′-endo/C3′-endo sugar pucker ($\delta = 90 \pm 10°$), compatible with those of B DNA, possibly due to the formation of a G-U wobble base pair. However, no significant misincorporation of guanine deoxynucleotides by the enzyme is detected with oligo(rU) as template. Mutual substrate displacement experiments show that the presence of templates and primer does not alter the relative affinities of the enzyme for complementary and noncomplementary substrates. Hence, a step subsequent to substrate binding and prior to DNA chain elongation is required to explain the high fidelity of template replication by Pol I. NOE studies reveal the average nucleotidyl unit of free oligo(rU)$_{54\pm11}$ to be of a high anti angle ($\chi = 70 \pm 10°$) with an O1′ ribose pucker ($\delta = 105 \pm 10°$). The binding of oligo(rU)$_{54\pm11}$ to the enzyme broadens the resonances of the oligonucleotide and slightly alters the average glycosyl angle ($\chi = 60 \pm 10°$), but the conformation remains B-like. The binding of oligo(rA)$_{50\pm13}$ to the enzyme broadens the resonances of the oligonucleotide and causes small downfield shifts of the adenine resonances consistent with decreases in base stacking as occur in the transition from the A to the B conformation. NOE studies reveal the average adenyl unit of enzyme-bound oligo(rA)$_{50\pm13}$ to be anti, ruling out the Z conformation. Intermolecular NOE's from proton resonances of the enzyme to those of the substrate analogue AMPCPP reveal the proximity of hydrophobic amino acids with chemical shifts over the range 0.6–1.8 ppm and an aromatic amino acid at 6.80 ppm, most likely a Tyr residue. Intermolecular NOE's from proton resonances of the enzyme at 0.80, 1.55, 1.92, and 3.05 ppm to those of the bound oligo(rU) and oligo(rA) templates are most simply explained by the proximity of cationic Arg and/or Lys residues and possibly a hydrophobic residue.

DNA polymerase (Pol I)[1] from *Escherichia coli* and its large fragment have been the subject of extensive study by the techniques of classical enzymology (Kornberg, 1980, 1982), X-ray crystallography (Ollis et al., 1985), NMR (Slater et al., 1972; Sloan et al., 1975; Ferrin & Mildvan 1985a,b), transient-state kinetics (Bryant et al., 1983; Mizrahi et al., 1985), and chemical and genetic modification (Joyce et al., 1985a,b) in order to learn the mechanism by which this enzyme catalyzes the accurate copying of DNA. The cloning of the large fragment of Pol I in a high-expression vector

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[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; NOE, nuclear Overhauser effect; PEI, poly(ethylenimine); pH*, meter reading in ^2H_2O; A/D, analogue to digital conversion; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; AMPCPP, adenosine 5′-(α,β-methylene-triphosphate); SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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