Transfer RNA structure by carbon NMR: C₂ of adenine, uracil and cytosine

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ABSTRACT

Fourier transform ¹³C NMR spectra of *E. coli* tRNA enriched with ¹³C in either position 2 of adenine (60 atom % ¹³C) or in position 2 of uracil (82%) and cytosine (63%) were taken at 25.16 MHz over the temperature range 100-76°. For C₂ of adenine the peak was initially 5 ppm wide, but narrowed to 0.5 ppm as the molecule unfolded. C₂ of uracil displayed behavior similar to that of adenine while the cytosine peak, initially relatively narrow at low temperature, sharpened less dramatically. Comparison of spectra at 26.16 MHz and 67.9 MHz showed that the peak widths for folded tRNA were determined largely by chemical shift non-equivalence. T₂ measurements suggested that intrinsic line widths of most cytosine C₂ peaks were 4 Hz and 2-3 Hz for uracil. Adenine C₂ with a directly bonded proton had resonances of about 40 Hz line width. T₁ values were measured for C₂ of adenine and the ribose carbons of tRNA. Consideration of dipolar relaxation and chemical shift anisotropy led to a calculated rotational correlation time of 1.6±0.4 x 10⁻⁸ sec for the adenines and 1.3±0.3 x 10⁻⁸ sec for the ribose carbons.

INTRODUCTION

For about a decade ¹³C spectroscopy has held the promise of supplying unique and valuable information on structure and dynamics of biological macromolecules. To a large extent the promise has been fulfilled for many proteins and enzymes (1,2). For nucleic acids the major problem of low sensitivity has inhibited progress. Early work on tRNA using natural abundance ¹³C NMR pointed out the potential of the technique for this macromolecule, but also made clear the tremendous problems of obtaining sufficient signal when quantities of material are limited (3,4).

Recent success in site-specific enrichment of *E. coli* tRNA with ¹³C (5,6) opens up a whole realm of experiments which were impossible before. In previous reports, we described investigations of tRNA enriched in ¹³C of methyl carbons on modified bases (7), and put forth the detailed protocol for maximal incorporation of ¹³C into *E. coli* tRNA from labeled precursors L - (methyl - ¹³C) methionine, (2 - ¹³C) adenine, and (2 - ¹³C) uracil (8).

Addition of the latter two bases yielded tRNA enriched in C₂ of A or C₂ of U and C. Our purpose in the present study is to examine in detail ¹³C NMR spectra of tRNA isotopically enriched in the 2-position of C, U and A. A major part of this work offers a comparison of spectra from the same sample taken at 2 different magnetic field strengths. Nuclear relaxation times were used to derive information on rotational reorientation of bases and ribose rings for tRNA in solution.

MATERIALS AND METHODS

Carbon 13 Enrichment of tRNA

E. coli C6 met⁻cys⁻rel⁻ was grown in stringently defined media as previously described in detail (8). Briefly, the media contained 6-mercaptapurine (10⁻⁵M) as an inhibitor of purine biosynthesis *de novo*. Added purines (0.054 mM adenine; 0.110 mM guanine) and pyrimidines (0.178 mM uracil; 0.180 mM cytosine) enabled the cells to overcome the drug block by utilizing the "salvage" pathways. Adenine enriched in carbon 13 at the C₂ position to the extent of 90+ atom % was the sole source of this base in some cultures; whereas uracil labeled to the same extent at C₂ was added to other cultures. The enriched precursors were obtained from Merck Canada and had been analyzed by ¹³C-NMR spectroscopy and mass spectrometry for position and extent of enrichment (5,8). Cells were collected in late log phase of the culture and tRNA was isolated as has been described (8). The position and extent of carbon 13 enrichment was determined from ¹³C-NMR of the tRNA and mass spectrometry of the tRNA bases obtained by acid hydrolysis followed by a high performance liquid chromatography separation (9). ¹³C₂-adenine enriched tRNA contained 60 atom % ¹³C₂-adenine. tRNA obtained from cultures grown with ¹³C₂-uracil was found to be enriched by 82 atom % ¹³C₂-uracil and 73 atom % ¹³C₂-cytosine. Uracil to cytosine conversion is possible by *E. coli* and is not blocked by 6-mercaptapurine which does inhibit adenine-guanine interconversions (8).

The tRNA preparations were checked for the amount of contaminating 5S RNA by urea-polyacrylamide gel electrophoresis (10). All preparations had less than 5% 5S RNA. Biological activity of all tRNA preparations was assessed by aminoacylation with each of 15 amino acids as catalyzed by an *E. coli* B extract (11). Approximately 1400 pmoles of amino acid was accepted per 50 µg (1 A₂₆₀ unit) of tRNA.

NMR Spectroscopy

All NMR spectra of the ¹³C-enriched tRNA preparations were acquired

from 1.4–2.8 mM solutions of tRNA that had been produced by extensive dialysis against glass distilled H₂O followed by concentration by evaporation under vacuum. Extensive dialysis was performed in order to observe conformational changes occurring under low Na⁺ and Mg²⁺ concentration.

Proton-decoupled ¹³C-NMR spectra of tRNA were obtained on a Varian XL-100 Fourier transform spectrometer (25.16 MHz for ¹³C) with a deuterium lock, Nicolet computer and Varian temperature control. tRNA solutions were 10% in D₂O in 12 mm diameter sample tubes. Other spectra, T₁, T₂ and nuclear Overhauser enhancement (NOE) measurements were taken with a high field spectrometer composed of a Bruker 63.4 kgauss magnet and 10 mm probe (67.9 MHz for ¹³C), Nicolet 1180 computer, quadrature detection transmitter-receiver, and a Bruker temperature control unit. Heating of the sample due to proton decoupling at 270 MHz was accounted for in determining the temperature. Dilute p-dioxane was used as an internal standard in the tRNA solutions with its signal assigned to 67.4 ppm relative to TMS.

T₁ values were measured by the inversion-recovery method with a wait time of at least 4 T₁'s between 180° pulses. Six or more points were used in a parameter computer fit of the data to an exponential recovery. T₂ measurements were done by the Carr-Purcell spin-echo sequence, without proton decoupling or with decoupling gated on for collection of the FID only. Estimated random error in T₁ values is ± 20% and ± 50% in T₂.

RESULTS AND DISCUSSION

Spectra of ¹³C₂-Enriched tRNA

Carbon NMR spectra of tRNA containing ¹³C₂-enriched adenine exhibited one major low field signal at 153 ppm (Figure 1A). Assignment was made from chemical shifts of C₂ of adenine, adenosine, AMP and poly A (7). The signal also contained small contributions (< 10% of its area) from natural abundance, non-protonated carbons in positions 4 of A, 4 and 2 of G and 2 of U. These signals appeared clearly in partially relaxed spin-echo spectra where the protonated adenine C₂'s decay away at least an order of magnitude faster than the non-protonated carbon peaks. Ribose carbons at natural abundance are clearly visible in this spectrum and are assigned in Figure 1A according to previous work (3,7).

Carbon NMR spectra of tRNA containing ¹³C₂-enriched uracil and cytosine exhibited two prominent low field signals at 151 and 157 ppm, respectively (Figure 1B). These signals were assigned from the known chemical shifts of C₂ of uracil, UMP and poly U, and CMP. A peak which we assign to C₂ of di-

Figure 1. Spectra of $^{13}\text{C}_2$ -enriched *E. coli* tRNA at 67.9 MHz. A) $^{13}\text{C}_2$ -enriched adenine sample, approximately 2.8 mM, in $\text{D}_2\text{O}/\text{H}_2\text{O}$, 30° . Spectrum represents 14,000 scans; 50° pulse of 8 μsec width and a pulse repetition time of 0.5 sec. Digital line broadening of 5 Hz was imposed on the free induction decay. Ribose carbons are identified in the 60-95 ppm range; d is p-dioxane, the internal standard, which was set to 67.4 ppm. B) $^{13}\text{C}_2$ -enriched cytosine and uracil tRNA, approximately 1.4 mM in $\text{D}_2\text{O}/\text{H}_2\text{O}$, 30° . Spectrum represents 10,000 scans using 50° pulses and a 1 sec pulse repetition time. Line broadening of 5 Hz was imposed on the free induction decay.

hydrouridine (4) is seen clearly at 155.5 ppm between the main C and U bands.

While the spectra of Figure 1 required 2-3 hrs. of signal averaging in order to bring out natural abundance detail, quite usable signals from the enriched carbons emerged after only 15-30 minutes. Enrichment in A, C or U provided an important practical advantage for ^{13}C studies of tRNA where sample quantities are limited, particularly if purified isoacceptor species are used.

NMR Frequency Dependence of Spectra

$^{13}\text{C}_2$ enriched samples were also investigated at 25 MHz, and Figure 2 shows a comparison of signals at 25.16 MHz and 67.9 MHz at 30° . Even though there is a difference of a factor of 2.7 in NMR frequency, the widths (in ppm) across the resonances are generally very similar when the same chemical species are compared, (i.e. the line widths in Hz are 2-3 times greater at

Figure 2. Comparison of C_2 resonances at 25.16 MHz and 67.9 MHz. Spectra are of dialyzed tRNA at 30 $^{\circ}$ in H $_2$ O/D $_2$ O. Spectra at 25.16 MHz were obtained with 4096 acquisitions, 90 $^{\circ}$ pulses of 40 μ s width and a pulse repetition time of 0.8 sec. Digital line broadening of 5 Hz was applied. The 67.9 MHz spectrum of the adenine C_2 enriched sample represents 14,000 scans, with 50 $^{\circ}$ pulses of 8 μ s width and a pulse repetition time of 0.5 sec; 5 Hz line broadening. The pyrimidine enriched spectrum required 10,000, 50 $^{\circ}$ pulses with a wait time of 1.0 sec; 5 Hz digital broadening. All spectra are displayed with the same scale in ppm where TMS = 0 referenced from internal dioxane at 67.4 ppm.

the higher field). This similarity could arise from one or both of two possibilities: 1) the resonance bands contain a spread of chemically shifted peaks and 2) the individual peaks are broader (in Hz) at the higher NMR frequency.

In order to help sort out the different effects, we made measurements of relaxation times and nuclear Overhauser enhancement (NOE) values at 68 MHz. The results are summarized in Table I where comparison is also made with line widths measured at 25.16 MHz. T_1 and NOE measurements are straightforward for ^{13}C -enriched tRNA. T_2 is another matter. Freeman and Hill (12) have summarized the pitfalls involved in spin-echo measurements particularly for coupled systems. They point out that noise modulated decoupling of protons can make it impossible to measure an accurate T_2 by the Carr-Purcell technique. We have therefore measured T_2 's of the C_2 -enriched tRNA's without ^1H decoupling, or with decoupling only during acquisition of the FID.

The results can be summarized as follows. At high temperatures (above 58°), in low Mg (II) and low salt concentrations, tRNA has lost its tertiary conformation and most of its secondary structure. The lines are narrow due to segmental motion. T_2 values approach, but do not equal T_1 's, as expected for flexible polymers. The NOE value for C_2 of adenosine, with a proton bonded to it, was still quite small, 1.33, again consistent with a flexible chain. NOE values of the non-protonated C_2 carbons of C and U were only 1.06 showing that dipolar relaxation did not dominate.

At 20° the macromolecule is mostly folded into a compact tertiary structure. ^{13}C NMR relaxation studies of a number of globular proteins (13) have led to some general expectations for molecules with correlation times like that of tRNA, at 68 MHz. 1) If a carbon has a directly bonded proton then its relaxation is dominated by dipole-dipole interactions; 2) Non-extreme narrowing conditions hold for "backbone" carbons and thus $\text{NOE} \approx 1.15$ and $T_1 \gg T_2$; 3) Line widths of individual peaks due to dipolar relaxation are somewhat narrower at 68 MHz than at 25 MHz for a proton-bonded carbon; 4) Chemical shift anisotropy, particularly for aromatic carbons, becomes an important relaxation mechanism for non-proton bonded carbons at high frequency, leading to broader lines and shorter T_1 values. In general these expectations are borne out for tRNA enriched in the aromatic C_2 of C, U, or A.

Referring to data at 20° in Table I it can be seen that NOE values are, within experimental error, equal to those predicted for a compact macromolecule and $T_1 \gg T_2$ in all cases as expected. The T_2 values are important for

Table I. ^{13}C Labeled tRNA. Results for T_1 , T_2 and NOE^a

	Temp.	ν_0 (MHz)	w_b (Hz)	T_2^c (sec)	T_1^d (sec)	NOE^e
C_2 Adenine	20°	25	116			
		68	262	.008	0.24	1
	32° (0.2M NaCl)	68	280	.006	0.36	
	58°	25	30			
		68	31		0.26	1.33
C_2 Cytosine	20°	25	22			
		68	58	(.01) ^f (.08)	1.1	1
	58°	25	17			
		68	37		1.1	1.06
C_2 Uracil	20°	25	78			
		68	245	.140	1.1	1
	58°	25	20			
		68	21		1.6	1.06
1' 2'(3') ^g Ribose 4' 5' (natural abundance)	32° (0.2M NaCl)	68		.007	0.35 0.34 0.37 0.18	

a. Unless otherwise indicated data are for tRNA solutions dialyzed against H_2O to minimize $\text{Mg}(\text{II})$ and NaCl concentrations.

b. Width at half height of resonance band

c. Estimated uncertainty of $\pm 50\%$

d. Estimated uncertainty of $\pm 20\%$

e. Ratio of peak area proton noise decoupled: undecoupled

f. 2 components of spin echo decay

g. 2' and 3' ribose carbon peaks overlap.

understanding the widths of spectral bands observed. For C_2 of adenine the width at 67.9 MHz is 262 Hz and 116 Hz for 25.16 MHz. However the average T_2 value of 8 msec corresponds to a line width of 40 Hz, far less than that actually observed, suggesting that the resonance band represents a spread of chemical shifts for individual peaks over a 3 ppm range. Assuming for the

moment that the individual peak line widths are NMR frequency independent, then the ratio of band widths in Hz (after subtracting 40 Hz) for 25.16 MHz vs. 67.9 MHz is 2.9, very close to the ratio of the two NMR frequencies (2.7), as would be expected if chemical shift non-equivalence were the source of the broader peak at higher field.

With C₂ of uracil and cytosine the spin-echo trains appear to have more than one relaxation time. For cytosine a fast component of about 30% of the amplitude has a T₂ of about 10 msec while the slow component relaxes with a time constant of 80 msec. With uracil the signal-to-noise ratio is not sufficient to resolve multiple relaxation rates if there are such. The peak has an overall T₂ of 140 msec, which corresponds to a line width of only 2.3 Hz. Particularly in the case of C₂ of uracil, the wide spread of chemical shifts combined with narrow intrinsic lines makes it likely that single carbon resonances will be identifiable in spectra of purified isoacceptor tRNA.

An increase in NMR frequency from 25.16 to 67.9 MHz did not significantly increase resolution for the C₂ resonance bands of A and C in unfractionated tRNA (Figure 2). For cytosine most of the individual lines are relatively narrow ($W_{1/2} \approx 4\text{Hz}$ based on the T₂ measurement), but there are too many overlapping peaks in a range of only 2 - 3 ppm. Use of purified isoacceptor tRNA may reveal single carbon resonances, however. With adenine the individual line widths are largely due to dipolar relaxation. Even though the peaks are spread over 5 ppm it seems unlikely that many A C₂'s will be resolvable in spectra of purified tRNA. One clear advantage of the high field magnet is in resolution of the dihydrouracil C₂ at 155.5 ppm (Figure 2B). The narrow line width probably reflects a high degree of mobility of this modified base which was noted earlier in other tRNA NMR studies (4, 14).

Temperature Dependence of Line Widths

Spectra of C₂ enriched tRNA were taken at 25.16 MHz over the temperature range 10° to 76°C. These samples had been dialyzed to lower the Mg and salt concentrations in order to spread out tertiary and secondary structure transitions (15). The adenine signal was 6 ppm wide at low temperature but sharpened considerably to 0.5 ppm when the tRNA was thermally denatured, as seen in spectra taken at 8 temperatures from 9.5° to 76.5° (Figure 3).

Spectra were taken of the U-C enriched tRNA preparation at the same 8 temperatures from 9.5° to 76.5°C (Figure 4). The signal assigned to C₂ of uracil was initially broad, however it sharpened with thermal denaturation in a manner similar to that of the C₂ of A. In contrast, the resonance signal from C₂ of cytosine was initially relatively narrow with an upfield

Figure 3. Temperature-dependence of the proton-decoupled Fourier transform carbon-13 NMR spectra of unfractionated *E. coli* tRNA (2.8 mM in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (1:1) specifically enriched with $^{13}\text{C}_2$ -adenine. Spectra were recorded at 25.16 MHz, with 4096 or 8192 acquisitions per spectrum. Chemical shifts are referenced to external TMS with dioxane (signal 5) used as the internal standard. Signal 1 is obtained from the C-2 position of every adenine residue; signal 2, from C-1 of ribose; signal 3, C-4 of ribose; signal 4, C-2 and C-3 of ribose; and signal 6, C-5 of ribose.

Figure 4. Temperature-dependence of the proton-decoupled Fourier transform carbon-13 NMR spectra of unfractionated *E. coli* tRNA (1.4 mM in H₂O/D₂O (1:1) specifically enriched with ¹³C₂-uracil. Spectra were recorded at 25.16 MHz, with 4096 or 8192 acquisitions per spectrum. Chemical shifts are referenced to external TMS with dioxane (signal 6) used as the internal standard. Signal 1 is obtained from the C-2 position of every cytosine residue, and signal 2 from the C-2 position of every uracil. Signal 3 from C-1 of ribose; signal 4, C-4 of ribose; 5, C-2 and C-3 of ribose; and 7, C-5 of ribose.

shoulder assigned to C₂ of dihydrouridine. Consequently, the cytosine-C₂ resonance did not exhibit as dramatic a narrowing as did that of uracil and adenine during thermal denaturation. The widths at half height of the pyrimidine signals are plotted in Figure 5 as a function of temperature along with the adenine C₂ data. Two major transitions are seen, one near 25° which probably reflects tertiary structure loss and one near 40-50° which would represent melting of helix regions.

As shown by the NMR frequency dependence, line widths in these spectra largely reflect chemical shift non-equivalence. We conclude that peak

Figure 5. Line widths at half height (in Hz) of C₂ resonance bands from Figures 3 and 4 are plotted vs. temperature. tRNA samples had been dialyzed against H₂O to minimize Mg(II) and NaCl concentrations in order to bring out tertiary structure transitions at low temperatures.

narrowing as the temperature increases represents a progressive loss of the non-equivalence due to loss of ordered structure. For C₂ of adenine, increased segmental motion when the tRNA melts leads to narrower intrinsic lines as well.

The behavior of the resonance bands for C₂ of U and A with increasing temperature seems quite reasonable in light of a large body of literature on thermal denaturation of tRNA. It is the behavior of the cytosine C₂ peak which is puzzling. A small decrease in line width occurs from 10° to 30° (25 Hz to 19 Hz) where tertiary structure is lost, and then essentially no change at all up to 76° in a region where A and U peaks decrease 7-fold in width. It seems likely that the modest change in cytosine line width with temperature is related to the relative narrowness of the peak initially at low temperature, compared to A and U. Apparently the chemical shift of C₂ of C is much less sensitive to details of RNA tertiary and secondary structure than are A and U. Work with complementary oligonucleotides and purified iso-acceptor tRNA's should help clarify these observations.

Rotational Correlation Times

If tRNA behaves in solution as a rigid rotor then we can use the meas-

ured T_1 value of protonated carbons to calculate a rotational correlation time, τ_R (16). In the presence of moderate concentrations of NaCl and below $\sim 35^\circ\text{C}$, tRNA's are generally folded into approximately their "native" tertiary structure with most of their bases stacked (16). In consideration of the crystal structure of yeast tRNA^{Phe} (17), all the adenines save the 3' terminus should be stacked, and should reorient approximately with the overall macromolecule. Proton NMR relaxation measurements made on adenine C₂ protons of tRNA^{Val}₁ (coli) confirm this expectation for the valine isoacceptor in 0.25 M NaCl below 40°C . (P. Schmidt and E. Edelheit, data to be published).

At 32° and in 0.2 M NaCl the T_1 value for C₂ of A in unfractionated E. coli tRNA is $0.36 \pm .07$ sec. If dipolar interaction with the C₂ proton is the only contributor to ^{13}C relaxation, then the two possible solutions for an isotropic rotational correlation time are $\tau_R = 1.4 \times 10^{-8}$ sec or 1×10^{-10} sec. The T_2 value of 6 msec is consistent only with the larger $\tau_R = 1.4 \times 10^{-8}$ sec, which is also the solution of choice based on consideration of the size of tRNA (17) and other measurements of rotational diffusion (18).

Chemical shift anisotropy (CSA) can contribute to relaxation of aromatic carbons at high magnetic fields (19). With a value of 200 ppm for the shift anisotropy, as is found for aromatic carbons of proteins (19), the contribution to $1/T_1$ of adenine C₂ is estimated to be 0.3 sec^{-1} . Additional dipolar relaxation from protons other than the directly bonded one, and from the two nitrogens adjacent to C₂ in the adenine ring was found to be negligibly important for $1/T_1$. Given the present uncertainties in the CSA contribution, we conclude that rotational reorientation of the adenine C₂ carbons (and thus the bases themselves) is described by $\tau_R = 1.6 \pm 0.4 \times 10^{-8}$ sec for 2 mM unfractionated E. coli tRNA in 0.2 M NaCl at 32° .

Ribose carbon peaks at natural abundance are easily seen in our spectra (Figure 1) because there are at least 75 of the furanose rings in each tRNA. Separate T_1 's were measured at 67.9 MHz for the 4 resonance bands containing 1', 4', 2'-3', and 5' ribose carbons, using the same sample with which the T_1 of adenine C₂ was measured (32° , 0.2 M NaCl). These data are collected in Table I. As expected, since it has two bonded protons, the 5' carbon longitudinal relaxation time (0.18 sec) is $\frac{1}{2}$ that of the other carbons. The other T_1 values (0.35, 0.34 and 0.37 sec for 1', 2'-3', and 4') are equal, well within the experimental error.

The T_2 value of 7 msec for 2'-3' was used to select the proper solution to the dipolar relaxation expression. This yielded a rotational correlation time of $1.3 \pm 0.3 \times 10^{-8}$ sec for the ribose carbons of tRNA at 32° , a value,

within experimental error, equal to T_R of the adenine rings.

Our τ_R calculations employ the commonly used C-H distance of 1.09 Å in the dipolar relaxation expression (19). Dill and Allerhand have recently pointed out that a small error in choice of this distance can lead to a large error in τ_R if the correlation time is near the " T_1 minimum" (20). Such is not the case here for our measurements at 63.4 kgauss, where relaxation is well into the non-extreme narrowing limit.

T_2 values of protonated tRNA carbons are consistently shorter (by about a factor of 2) than expected based on the T_1 data. Systematic errors (12) might account for this discrepancy as could additional transverse relaxation mechanisms such as chemical exchange. Line widths of resolved peaks in purified, single species of tRNA will help clarify the T_2 question. For the present study T_2 's serve as a qualitative guide to choosing the appropriate solution of the relaxation equation, and also serve to suggest what values of line widths can be expected when single carbon resonances are resolved.

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