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Carbon-13 NMR studies on [4-^{13}C] uracil labelled E. coli transfer RNA$_I^{Val}$[1]

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ABSTRACT

In this paper we describe carbon-13 nuclear magnetic resonance results on ^{13}C-enriched purified transfer RNA$_I^{VAL}$ from E. coli SO-187, a uracil requiring auxotroph. The organism was grown on uracil 90% ^{13}C-enriched at the carbonyl C_4 position. Transfer RNA$_I^{Val}$ was purified from bulk tRNA by sequential chromatography on columns of BD cellulose, DEAE-Sephadex A-50 and reverse gradient sepharose 4B. Dihydrouridine, 4-thiouridine,[3] and uridine 5-oxyacetic acid located at discrete positions in the polymer backbone were tentatively assigned in the highly resolved 25 MHz ^{13}C-spectra. Chemical shift versus temperature plots reveal differential thermal perturbation of the ordered solution structure, evident in the large dispersion (ca 3-4 ppm) of the uridine C_4 resonances. Over the range 26-68°C, V in the anticodon displays the largest downfield shift. Whereas several uridine residues rapidly shift downfield between 50-68°, one moves upfield beginning at 37°. The results are qualitatively compared with proton NMR analysis of the three dimensional structure.

INTRODUCTION

Of the many spectroscopic techniques which have been utilized to probe the structural and dynamic properties of transfer RNA in solution, nuclear magnetic resonance has been quite illuminating. In particular, monitoring lowfield protons involved in secondary and tertiary hydrogen bonding interactions has yielded many insights and has provided a comparison with the solid state.[4-12] Despite the success of this approach, there exists the difficult problem of definitively assigning the resonances from 23-30 imino and amino protons involved in secondary and tertiary hydrogen bonds.[4]

Carbon-13 NMR studies of transfer RNA site specifically enriched in carbon-13 offers advantages in ease of assignment, a broad chemical shift range and the potential to readily relate relaxation data to specific dynamic features of the molecule. In addition, monitoring only the ^{13}C-enriched carbons overcomes the peak overlap and sensitivity difficulties of the pioneering natural abundance

studies of Komoroski and Allerhand.[13,14] Several reports on bulk transfer RNA enriched *in vivo* with carbon-13 have recently appeared. Agris and co-workers [15-17] have utilized the *E. coli* methionine auxotroph C6 to obtain tRNA labelled in the various methyl groups of modified nucleosides and also to incorporate ^{13}C-2 labelled uracil and ^{13}C-2 labelled adenine into tRNA. Hamill, et al. [18,19] have also used a nutritional auxotroph to incorporate 90%[4-^{13}C]-uracil into tRNA of *Salmonella typhimurium* JL-1055, a uracil requiring strain which was selected in order to insure good incorporation and avoid the side labelling of cytidines. The carbonyl carbon-4 was chosen as a site for labelling because: 1) It is a quaternary carbon with a fairly long T_1, (1.5-3 sec; Hamill, et al.[19]) thus having a narrow resonance; 2) It is involved in secondary and tertiary structure hydrogen bonding; 3) It has been found to be a probe sensitive to the electronic and magnetic environment around it (Hamill, et al.[18]). 4) Most of the unusual uracil nucleosides in tRNA involve modification at C_5 thus affording the opportunity to identify and monitor these unusual residues as well as uridine itself.

Ideally, one would want to study invidividual isoaccepting tRNA molecules in order to correlate ^{13}C NMR parameters with molecular structure and dynamics and to relate these properties with biological features. In this communication we describe encouraging 25 MHz carbon-13 NMR data on $tRNA_I^{Val}$ purified from bulk tRNA isolated from the uracil requiring *E. coli* strain SO-187 grown on 90%[4-^{13}C]uracil. To our knowledge, this is the first report on a specific isoaccepting tRNA containing C-13 enrichment at known positions in the polymer chain.

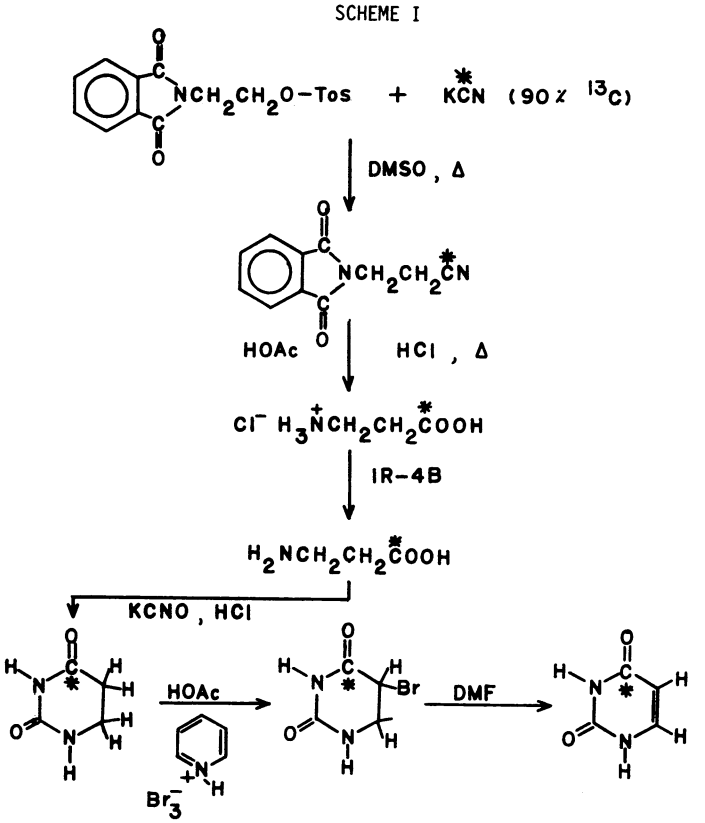
METHODS

Preparation of [4-^{13}C]-uracil labelled $tRNA_I^{Val}$

E. coli SO-187 was obtained from Dr. Jan Neuhard. This strain requires histidine, leucine, methionine, cytidine, thymidine and uracil for normal growth which was accomplished in minimal media of glucose and salts supplemented with the required nutrients including 90%[4-^{13}C]-Uracil. This C_4 labelled uracil was prepared in 17% overall yield based on KCN via Scheme I (asterisk denotes ^{13}C enrichment).

A total of 2.2 Kg of cell paste yielded 4.7 gms bulk tRNA which was obtained by the standard phenol extraction methods, modified to include precipitation of tRNA with 2 M LiCl[20] and final purification on DEAE-Sephacel. The tRNA was stripped of endogeneous amino acids by incubating in 2 M tris. HCl, pH8 for 90 minutes followed by ethanol precipitation. $tRNA_I^{Val}$ was obtained from 1.4 g

SCHEME I



of the bulk tRNA using a modification of the method of Reid, et al.[21]. We were unable to obtain homogeneous $tRNA_I^{Val}$ following chromatography on BD cellulose (Cellex BD from Bio-Rad) and DEAE-Sephadex A-50 (Pharmacia 40-120 μ or Bio-Rad DEAE Bio-Gel A) as reported by these authors. Partially purified $tRNA_I^{Val}$ (^{13}C-valine acceptance; 700 pmole/A_{260}) as obtained from the DEAE-Sephadex A-50 column was chromatographed on the third type of column suggested by Reid, et al.[21], Sepharose 4B using a reverse salt gradient.[22] Homogeneous $tRNA_I^{Val}$ was obtained between 0.85 and 0.75 M $(NH_4)_2SO_4$, eluting later than methionine, glycine and glutamic acid activities. The ^{14}C-valine acceptance was greater than 1400 pmoles/A_{260}. For the charging assays we used partially purified valine synthetase obtained from DEAE chromatography as described by LaPointe and Soll.[23]

Slow scanning mass spectrometry was performed on TMS derivitized uridine

obtained from $tRNA_I^{Val}$ by digestion with ribonuclease T_2 and bacterial alkaline phosphatase. Per-^{13}C-labelled uridine was used as a carrier. Analysis of the M + 1 peak (selected ion monitoring not performed) indicated that 75% of the uridines are ^{13}C enriched whereas only 2-3% of the cytidines are labelled.

NMR Measurements

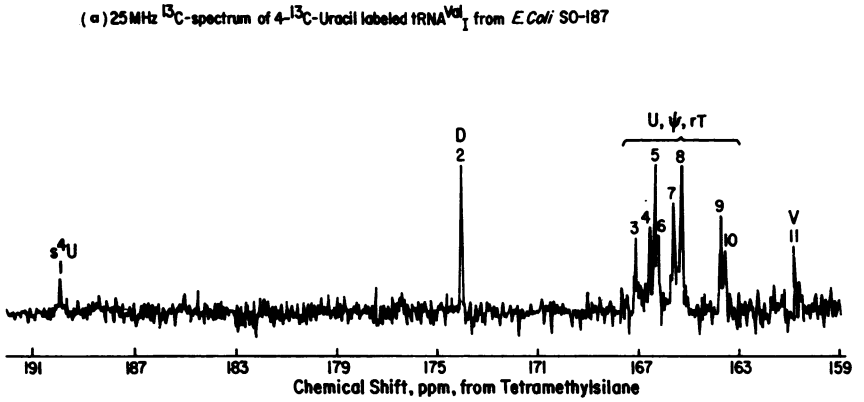
Two separate isolations of the [4-^{13}C]uracil labelled $tRNA_I^{Val}$ were carried out. In the first, seven mg lyphilized solid were isolated and used to produce the ambient temperature ^{13}C spectrum in Figure 1. The second larger isolation yielded 25 mg which was used for the thermal perturbation studies (Figure 2). The $tRNA_I^{Val}$ samples were dissolved in 0.3 ml phosphate buffered D_2O solutions (pD = 7.3) containing NaCl, $MgCl_2$, EDTA, $Na_2S_2O_3$, and NaN_3 as given in the figure captions. D_2O solvent was used to provide a strong lock signal. These solutions in 5 mm tubes were examined at 25 MHz using a Varian XL-100 spectrometer operating in the FT mode. Chemical shifts were measured from internal dioxane and converted to TMS by adding 66.3 ppm. A standard Varian variable temperature accessory was used for the thermal perturbation work. Probe temperatures were measured by a precision thermometer inserted into the sample spinner to the normal sample depth in the probe.

RESULTS AND DISCUSSION

Resonance Assignments

The 25 MHz ^{13}C-spectrum of the [4-^{13}C]uracil enriched $tRNA_I^{Val}$ is displayed in Figure 1(a). Individual chemical shifts and tentative assignments are in Table I. The well resolved ambient temperature spectrum contains eleven resonances of the fourteen which would be expected based upon the sequence of *E. coli* K12 $tRNA^{Val}$.[24,25] Peaks 5, 7 and 8 are thought to consist of overlapping resonances. The nuclear Overhauser enhancements (NOE's) of the C_4 in dihydrouridines and uridines in tRNA have been reported to be respectively 1.5 and 1.1[19]. This larger NOE for the C_4 of dihydrouridine is sufficient to account for the larger size of this line.

Assignments listed in the table for several of the C_4 signals from modified uridine nucleosides were based on comparison with reference nucleosides. Preliminary studies on the binding of paramagnetic manganese and cobalt ions reinforce the assignment of peak #11 to V. Also preliminary results on [4-^{13}C] uracil labelled $tRNA^{phe}$, which does not contain V, shows no resonance at 160 ppm. In agreement with previous x-ray data on yeast $tRNA^{phe}$ [26] and lowfield ^1H-NMR on *E. coli* $tRNA_I^{Val}$ [9] we find that both ions bind in the hinge region of the tRNA



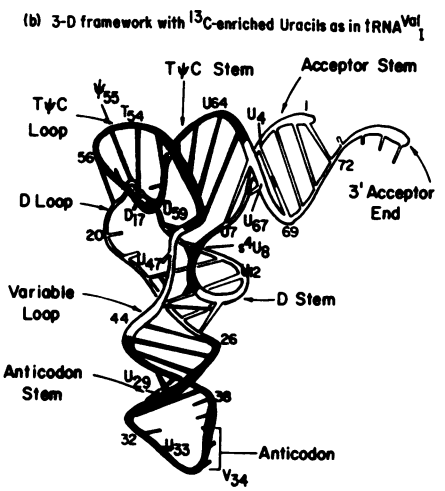


Figure 1(a). 25 MHz spectrum of [4-^{13}C]uracil labelled $tRNA^{Val}_I$. 21 Mg/ml in D_2O solution of 30 mM K_2HPO_4(pD - 7.3), 150 mM NaCl, 15 mM $MgCl_2$, 1.5 mM EDTA, 3 mM $Na_2S_2O_3$, 0.03% NaN_3; ambient temperature 32-34°C. 1 KHz sweep width, 16,304 transients, 90° pulse (45 μsec), and 4 second acquisition time. Chemical shifts measured from internal dioxane and converted to TMS by adding 66.3 ppm. (b). Three dimensional framework of yeast $tRNA^{Phe}$ with the superposition of the ^{13}C enriched uracils as found in E. coli $tRNA^{Val}_I$.

three dimensional structure (see Figure 1(b)) by noting the preferential intensity loss at s^4U and D as well as other peaks in the uridine region with progressive addition of metal. However, these titrations had negligible effect on peaks 4, 7 and 11 which indicates these residues are located some distance

TABLE I. ^{13}C-Chemical Shifts (in ppm from TMS) of C_4 Carbonyls from Reference Nucleosides and 4-^{13}C-uracil Labelled *E. coli* SO-187 $tRNA_I^{Val}$.

Peak (see Fig. 1)	Chemical Shift (ppm)	Assignment	Reference Chemical Shift (ppm)
1	189.2	4-Thiouridine (s^4U)	190.9
2	173.4	Dihydrouridine (D)	173.5
3	166.6		
4	166.0		
5	165.7	Ribothymidine (rT)	166.0 (rT)
6	165.6	Uridine (U)	165.8 (U)
7	165.1	Pseudouridine (ψ)	165.0 (ψ)
8	164.8		
9	163.3		
10	163.1		
11	160.4	Uridine-5-oxyacetic Acid (V)	161.4

from the "bend" area, i.e., in the anticodon loop (V,U_{29}, U_{33}) or acceptor stem (U_4).

Thermal Perturbation Studies

It is of interest to note the range of shifts for the uridines in the tRNA (163-167 ppm) compared with ∿166 ppm for uridine itself. This is indicative of the influence of tRNA secondary and tertiary structural effects upon the environment of the individual uridine residues. Hamill, et al[18,19] have shown that this nonequivalence disappears upon heating through the order-disorder transition. Figure 2 is a plot or resonance position versus temperature. The data were collected sequentially from 31-68°C, followed by the data at 26.5°C. The fact that the peak positions at this low temperature fit nicely with the other data argues for the reversibility of structure and conformation.

One can readily note the numerous differential thermal effects in Figure 2. "V" in the anticodon loop displays twice the downfield shift (∿1 ppm) over the temperature range as do the other peaks. At 31°C, peak #5 splits and at 36.5°C peak, 6b appears to the high field side of #6. This resonance displays a steady upfield movement with temperature increase, contrary to every other resonance. Between 49-62°C peak #7 yields two resonances and #8 splits into three separate lines. At 62°C 15 discrete resonances can be counted, one more

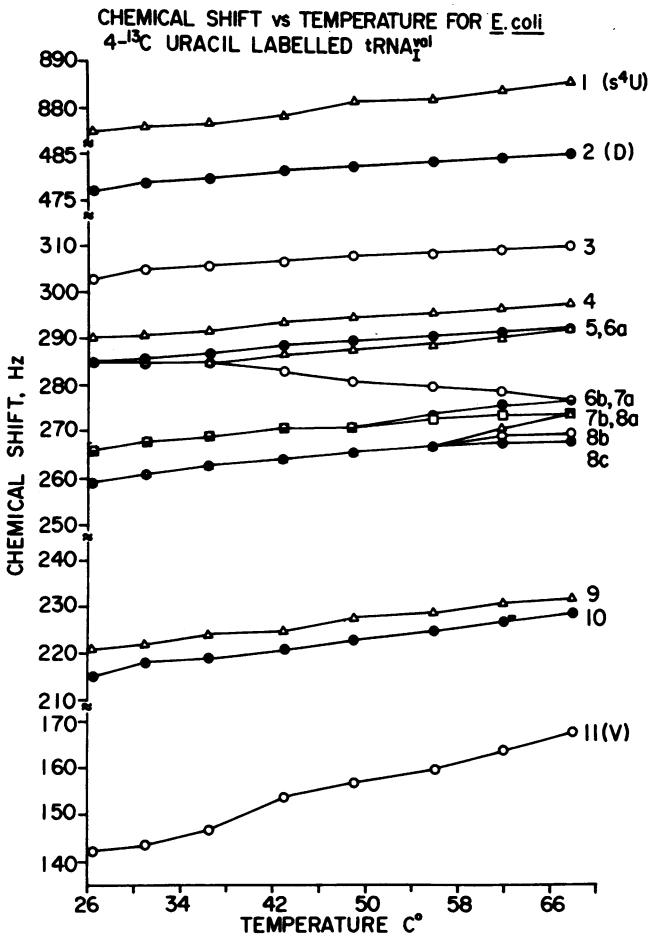


Figure 2. Chemical shift versus temperature for the C_4 carbonyl resonance of [4-^{13}C]uracil labelled $tRNA_I^{Val}$. 75 mg/ml in a D_2O solution of 20 mM K_2HPO_4 (pD=7.3), 100 mM NaCl, 10 mM MgCl, 5 MM EDTA, 2 mM $Na_2S_2O_3$, 0.02% NaN_3. 1 KHz sweep width, 15-20,000 transients per spectrum. Dioxane in this milieu shifted ca 10 Hz downfield over the temperature range.

than the 14 expected from the E. coli B of K12 primary sequence.[24,25] Either this mutant has an extra uridine residue, or the modified uridines are not 100 per cent modified.

In addition to these differential shift changes, the s^4U resonance narrows at 36.5°C, indicating a possible rupture of the tertiary structure involving the s^4U and A_{14} bases. This change in the s^4U line width supports the explanation

that the low intensity and broad base of this line is due to the restriction of motion experienced by this nucleoside in its tertiary environment. Reid's group has recently published proton NMR results on the low field exchangeable imino and amino protons involved in tertiary and secondary hydrogen bonds for several purified transfer RNA's.[6-9] They show that in $tRNA_I^{Val}$, (refer to Figure 1(b)) tertiary interactions involving rT_{54}-A_{58} and s^4U_8-A_{14} are weakened early as temperature is raised, as well as is G_{18}-ψ_{55}.

It is not strictly possible to directly compare the low field proton NMR data with our own because of the fact that our samples are dissolved in D_2O as opposed to water. However, we have found that replacing H_2O for the D_2O yields essentially the same spectrum at 32-34°C. D_2O was, as mentioned above, used to provide a strong lock signal, particularly for the variable temperature studies. It is probable that the perturbations seen here at 49-62°C reflect the general breakup of tertiary structure involving the D- and TψC loops (Figure 1(b)).

The appearance of peak #6 and the line narrowing for s^4U at 31-37°C seem to indicate an early structural perturbation and perhaps rearrangement of tertiary structure leading to a more flexible segment. On this basis U_7, adjacent to s^4U_8, may give rise to peak #6.

The gradual upfield drift of 6b undoubtedly reflects the greater time average diamagnetic influence of neighboring purine rings as the tRNA structure is perturbed. We do not know at this time which uridine residue is so affected, but it is probably in the "bend" region of the molecule. In the case of V in the anticodon, the larger downfield movement may signal a gradual lessening of diamagnetic ring current effects from purines in the anticodon loop (A_{36}, m^6A_{37}, A_{38}).

We are continuing our assignment efforts of the various uridine C_4 resonances. These include specific nucleolytic fragmentation. Purification of other specific tRNA's is also underway. The results reported here demonstrate the considerable promise of carbon-13 NMR as a means of probing the structure and conformation of purified ^{13}C-enriched transfer RNA.

REFERENCES

1. Presented at the 21st Rocky Mountain Conference on Analytical Chemistry Biopolymers Symposium, Denver, July 30, - August 1, 1979. This work was supported by USPHS Grant GM 25512 (MPS) and GM08421, RR07092 and RR00574 (DMG). One of the authors (WDH) is indebted to the NIH for support in the form of Postdoctoral Fellowship GM 05546. We are grateful to Dr. Susumu Nishimura, National Cancer Research Center, Tokyo, for a generous sample

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2. Address correspondence to this author. Phone 801-581-7599.
3. Abbreviations used: uridine (U); 4-thiouridine (s^4U); dihydrouridine (D); ribothymidine (rT); pseudouridine (ψ); uridine-5-oxyacetic acid (V); tetramethylsilane (TMS); T_1; spin-lattice relaxation time; deuterium oxide (D_2O).
4. Bolton, P. H. and Kearns, D. R. (1978) in Biological Magnetic Resonance, Eds. Berliner, L. J. and Reuben, J., Plenum Press, pp. 91-137.
5. Patel, D. J. (1978) Ann. Rev. Phys. Chem. 29, 337-362.
6. Reid, B. R., Ribeiro, S. N., McCollum, L., Abate, J. and Hurd, R. E. (1977) Biochemistry 18, 3996-4005.
7. Hurd, R. E. and Reid, B. R. (1979) ibid 18, 4005-4011.
8. Hurd, R. E. and Reid, B. R. (1979) ibid 18, 4017-4024.
9. Hurd, R. E., Azhderian, E. and Reid, B. R. (1979) ibid 18, 4012-4017.
10. Johnston, P. D. and Redfield, A. G. (1979) in Transfer RNA, Part 1, Structure, Properties and Recognition, Eds. Schimmel, P., Soll, D. and Abelson, J. Cold Spring Harbor Laboratory Press, pp. 181-206.
11. Robillard, G. T., Tarr, C. E., Vosman, F. and Berendsen, H.J.C. (1976) Nature 262, 363-369.
12. Robillard, G. T., Tarr, C. E., Vosman, F. and Reid, B. R. (1977) Biochemistry 16, 5261-5273.
13. Komoroski, R. A. and Allerhand, A. (1972) Proc. Nat'l Acad. Sci. USA, 69, 1804-1808.
14. Komoroski, R. A. and Allerhand, A. (1974) Biochemistry 13, 369-372.
15. Agris, P. F., Fujiwara, F. G., Schmidt, C. F. and Loeppky, R. N. (1975) Nucl. Acids Res. 2, 1503-1572.
16. Tompson, J. G., Hayashi, F., Pankstelis, J. V., Loeppky, R. N. and Agris, P. F. (1979) Biochemistry 18, 2070-2085.
17. Tompson, J. G. and Agris, P. F. (1979) Nucleic Acids Research 7, 765-779.
18. Hamill, W. D., Jr., Grant, D. M., Horton, W. J., Lundquist, R. and Dickman, S. (1976) J. Amer. Chem. Soc. 98, 1276-1278.
19. Hamill, W. D., Jr., Horton, W. J. and Grant, D. M. (1979) ibid, submitted 1979.
20. Avital, S. and Elson, D. (1969) Biochim. Biophys. Acta. 179, 297-307.
21. Reid, B. R., Ribeiro, S. N., McCollum, L., Abate, J. and Hurd, R. E. (1977) Biochemistry 16, 2086-2094.
22. Holmes, W. M., Hurd, R. E., Reid, B. R., Rimerman, R. A. and Hatfield, G. W., (1975) Proc. Nat'l Acad. Sci., USA 72, 1068-1071.
23. LaPointe, J. and Soll, D. (1972), J. Biol. Chem. 247, 4966-4974.
24. Harada, F., Kimura, F. and Nishimura, S. (1971) Biochemistry 10, 3269-3276.
25. Harada, F., Kimura, F. and Nishimura, S. (1971) ibid 10, 3277-3283.
26. Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S. and Klug, A. (1977) J. Mol. Biol. 14, 315-328.