NMR Study of Nitrogen-15-Labeled *Escherichia coli* Valine Transfer RNA†

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ABSTRACT: 1,3-^{15}N-Labeled uracil was synthesized chemically and used to prepare labeled *Escherichia coli* tRNA^{Val} biosynthetically. 500-MHz measurements of ^{15}N and proton chemical shift were obtained, for all uridine and uridine-related bases, by heteronuclear multiple-quantum coherence spectroscopy. All the uracil NH group resonances were assigned and were in agreement with previous proton-only assignments. The temperature dependence of intensities of resonances was used to infer the relative stability of parts of the molecule. The acceptor stem was the least thermally stable structural feature, while the anticodon and T loop were relatively more stable.

Transfer RNA was for many years a test molecule for development of NMR methods, such as early NOE assignment strategies, selective pulses for water elimination, and proton exchange rate measurements [reviewed by Reid (1981)]. Structural results obtained by these methods included, for example, direct evidence of the existence of stable nonstandard base pairs, such as the GU pair, and a triple base interaction (Choi & Redfield, 1985). Some of the first distance estimates by NOE in a macromolecule were performed on tRNA's, and the first large-scale NOE assignment of a nucleic acid also was accomplished on this molecule (Roy & Redfield, 1981).

From the beginning, a goal of this research was to determine approximate rates of unfolding of tRNA and also to estimate relative stabilities of tertiary interactions compared to stabilities of the four stems of tRNA, as a function temperature (Johnston & Redfield, 1981a). This work was hampered by the severe overlap of the one-dimensional spectra which were used and also failed to take into account the possibility that the rate-determining step in imino proton exchange might be the exchange from open unpaired bases, rather than the rate of opening of the secondary or tertiary interaction (Leroy et al., 1985b).

Work on tRNA's also stimulated the first development of heteronuclear multiple-quantum coherence (HMQC)[1] NMR spectroscopy in biopolymers (Bax et al., 1983). Here we combine some of these methods to eliminate the overlap problem and to illustrate simple methodology for investigation of dynamics of *Escherichia coli* tRNA^{Val}, by studying the temperature dependence of the HMQC peaks in a transfer RNA that is regiospecifically labeled with [^{15}N]uracil. Development in this field continues rapidly, most significantly with the recent demonstration that larger RNA molecules can be fully labeled with both ^{15}N and ^{13}C and analyzed by 3D NMR (Nikonowicz & Pardi, 1992). Nevertheless, simple measurements of the type presented herein will still remain useful for dynamic studies on larger nucleic acids even when identifications are performed with modern multiple labeling NMR methods.

MATERIALS AND METHODS

Chemical synthesis of [^{15}N]uracil was achieved as described, starting from [1,3-^{15}N]urea (Roy et al., 1984; Lipnick & Fissekis, 1979). Labeled uracil (1.3 g) was used in a 100-L fermentor with a yield of typically 250 g of cells and a final yield of 90 OD_{258} units of *E. coli* tRNA^{Val}, having activity of at least 1.6 nmol/A_{258} unit. The auxotrophic strain CGSC 4501, kindly provided by Dr. B. Bachman of the *E. coli* Genetic Stock Center, Yale University, was used, and the tRNA was isolated as described (Sanchez et al., 1980). Labeled tRNA (90 units) was dissolved in 160 μL of buffer containing 20 mM EDTA, 10 mM $NaPO_4$, and 0.1 M NaCl, adjusted to pH 7, and dialyzed three times in a flow microcell against 100 mL of buffers that were the same except that EDTA was 1 mM and 6% D_2O was included in the final dialysis. Samples of C8 deuterium-labeled and unlabeled *E. coli* tRNA^{Val} were produced by similar methods.

We used previously described NMR methods for water signal elimination, nuclear Overhauser effect (NOE), and ^{15}N HMQC spectroscopy (Griffey et al., 1983; Roy et al., 1984). In some cases, internuclear double resonance (INDOR) difference decoupling one-dimensional proton NMR was also used with low-power ^{15}N decoupling whose frequency was varied, to determine ^{15}N shifts [see, for example, Roy et al. (1984)].

RESULTS

The one-dimensional NMR spectrum was virtually identical to that reported by Hare et al. (1985), provided ^{15}N decoupling was applied during the free induction decay (not shown). HMQC spectra were obtained from 19 to 39 °C. Temperature variations of HMQC chemical shifts were nearly zero within experimental error (0.1 ppm ^{1}H, 0.4 ppm ^{15}N) from 19 to 39 °C, and the main temperature effect was loss of intensity of some peaks. Representative 2D spectra are shown in Figure 1, and the sequence, secondary structure, and presumed tertiary structure of *E. coli* tRNA^{Val}, based on the yeast tRNA^{Phe} crystal structure, is shown in Figure 2 for the reader's convenience.

Chemical shifts for uracil ^{15}NH resonances are summarized in Table I. Most resonance identifications were made independently from the previous assignments of Hare et al. (1985), and in some cases by different methods, but are in complete agreement with those assignments. The acceptor stem pairs AU4, 6, and 7 were assigned by sequential one-

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[1] Abbreviations: D, dihydrouridine; HMQC, heteronuclear mutliple-quantum correction spectroscopy; INDOR, internuclear double resonance difference decoupling; Ψ, pseudouridine; V, uridine 5-oxyacetic acid.

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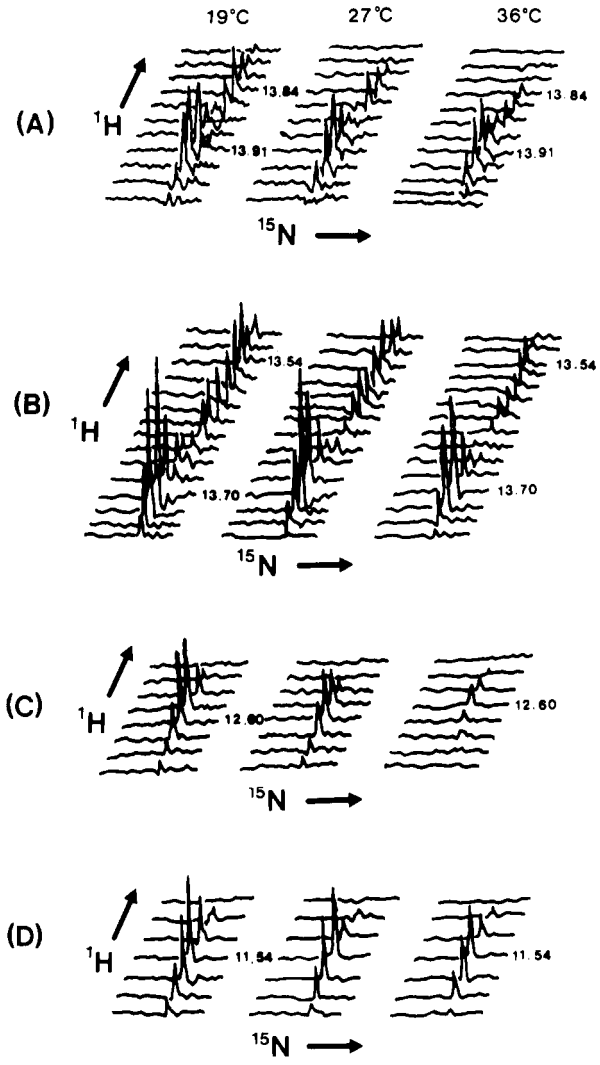

FIGURE 1: Parts of the HMQC spectra of [^{15}N]uracil-labeled *E. coli* tRNA^{Val} at different temperatures. Sections of a stacked plot are shown, to best show the temperature dependence of the resonances. The spacing between lines in the proton dimension is 10 Hz. The total width in the ^{15}N dimension is 1 kHz, and each spectrum was obtained in 5 h, with 512 by 128 complex input points, zero-filled to double this in both dimensions. (A) AU29 and AU12; (B) T54-A58 and AU4; (C) AU7; (D) Ψ55 ^{15}N3H.

dimensional NOE using labeled or unlabeled samples (Choi, 1984). The remaining standard AU pair identifications 12 and 29 are confirmed, as judged by their normal ^{15}N shifts, and their specific identities are based on the previous assignments (Hare et al., 1985). The two protons of GU50 were assigned by their characteristic mutual NOE (Johnston & Redfield, 1981a) and by the existence of an HMQC peak for only U64, since G50 contained no label. The resonances of Ψ55 were assigned as described previously for other tRNA's (Tropp & Redfield, 1981; Roy & Redfield, 1983). The resonances of the Hoogsteen paired bases T54 and s^{4}U8 were collectively assigned by lack of an aromatic NOE in a purine C8-deuterated tRNA compared to an unlabeled sample (Sanchez et al., 1980), and s^{4}U8 was then assigned from its unusual ^{15}N shift. A single resonance at 9.98 ppm (proton) is likely to arise from a uracil that is not internally base paired. We could find no one-dimensional NOE partners for this peak. The NH resonances of dihydrouridine (D17) and uridine 5-oxyacetic acid (V34) are likely to be shifted, in the ^{15}N dimension, away from the normal uridine position around 155 ppm, because of the covalent modifications of these rings. Therefore, this resonance is not likely to arise from these bases,

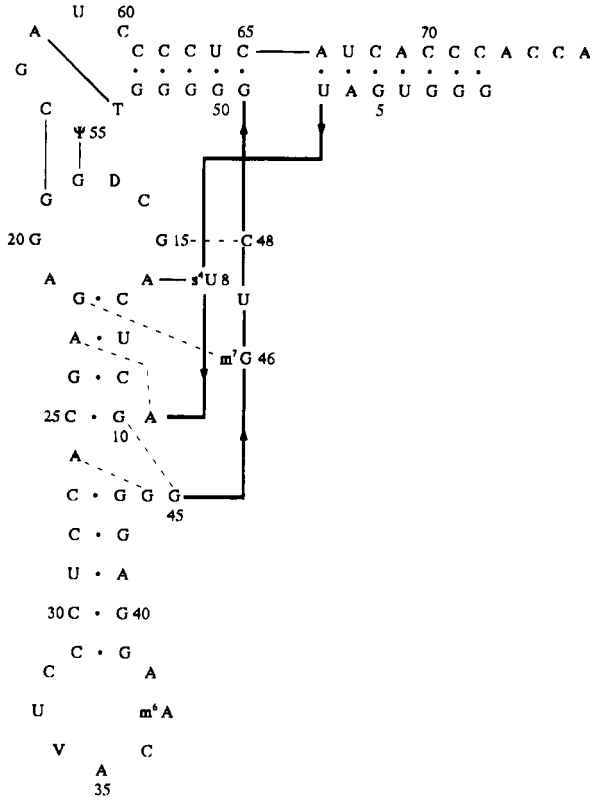

FIGURE 2: Nucleotide sequence of *E. coli* tRNA^{Val}. The sequence is shown in the form introduced by Kim (1978) to emulate the tertiary structure of yeast tRNA^{Phe}.

Table I: Assignments for [^{15}N]Uracil-Labeled *E. coli* tRNA^{Val} at 27 °C

peak[a]	assignment	^{1}H[b] (ppm)[c]	^{15}N (ppm)[d]
A	s^{4}U8A14	14.9	182.1[e]
B	AU6	14.35	163.1
CD	AU29	13.91	157.9
CD	AU12	13.84	162.2
F	T54A58	13.7	156.2
G	AU4	13.54	160.9
O	AU7	12.6	156.3
T	GU50	11.98	152.4
VW	Ψ55N3	11.54	156.3
X	Ψ55N1	10.97	137.7[e]
Z	U59(?)	9.98	152.3

[a] The peak designations are those of Hare et al. (1985). [b] All assignments are for the uracil (U), pseudouridine (Ψ), or thymidine (T) N3H groups except as indicated. [c] The ^{1}H shifts are expressed relative to DSS. [d] The error is ~0.4 ppm except as marked, obtained by HMQC. [e] The error is ~1.5 ppm, obtained by INDOR at 270 MHz, 15 °C.

and the remaining candidates for its assignment are the N3H group of U33 or of U59. We tentatively assign the 9.98 ppm resonance to U59, because this base appears to be relatively buried in the T loop in the yeast tRNA^{Phe} structure.

HMQC peaks from paired uracils that are not shown in Figure 1 showed no clear intensity variation with temperature, except for that of AU6 which was similar to AU4 and AU7 (Choi, 1984). The resonance of T54 was noticeably stronger than the others, by a factor of about 2, perhaps because of a low value of the likely major broadening interaction for these resonances, which is proton chemical shift anisotropy. A similar strong T54 HMQC resonance has been observed in other tRNA's (Griffey et al., 1983; Roy et al., 1984).

DISCUSSION

We have confirmed the previous proton assignments (Hare et al., 1985) for uracil N1H resonances in *E. coli* tRNA^{Val}

and augmented or strengthened them by confirming the GU50 specific assignments, by providing ^{15}N shifts, and by observation of a single noninternally bonded uracil resonance at 9.98 ppm.

To be observable at nearly full intensity by HMQC, a ^{15}NH group must have an effective T_2 that is somewhat greater than twice the HMQC evolution time τ. The time τ was 3.5 ms in our experiments. The HMQC peak volume will be decreased by a factor $\exp(-2\tau/T_2)$ by relaxation during the two τ intervals, where T_2 is very nearly the single-quantum proton T_2, or the inverse of π times the proton line width. Somewhat less important is relaxation during the evolution times, which affects peak heights and widths without affecting their volumes. The overall rate T_2^{-1} is the sum of a relaxation rate due to chemical shift anisotropy and magnetic dipolar interaction, and the rate of chemical exchange rate of proton exchange with solvent. The first rate contributes about 60 s^{-1} T_2^{-1}, for most of the protons reported in Table I, judging from their low-temperature proton line widths of about 20 Hz. Disappearances, or intensity decreases, of resonances which are reported here are assumed to be due to a contribution of solvent exchange at a rate of 60 s^{-1} or more, to the overall rate. Another possible way that the relaxation rate could increase is as a result of slow conformation change at more than this rate, but there is no evidence for such a change.

The single strong uracil peak observed at 9.98 ppm must therefore exchange with solvent slowly. As indicated above, there are two candidates for this peak, U59 and U53. Of these, the one that is not found in our spectra, most likely U33, must exchange with solvent at a rate of about 100 s^{-1} or faster, while the other one, tentatively U59, must exchange much more slowly. An imino proton of an unpaired base is expected to exchange at a rate which is buffer dependent but is much greater than 100 s^{-1} [see, for example, Leroy et al. (1986)]. We did not find resonances that could be assigned to D17 and V34, and we presume that these bases are at least partially solvent-exposed. On the other hand, we did observe the resonance of the Ψ55 N1H group which is not internally hydrogen bonded (Tropp & Redfield, 1981). Similar resonances of pseudouridines involved in secondary and tertiary interactions in transfer and other RNA's have been observed and discussed elsewhere [see, for example, Roy and Redfield (1983), Griffey et al. (1985), and Hall and McLaughlin (1992)].

The chemical shifts reported in Table I are as expected from work on other tRNA's except for the exceptionally far upfield AU7 resonance at a proton shift of 12.6 ppm. There are no other secondary AU NH resonances observed in RNA or DNA more upfield than 13 ppm, to our knowledge, other than the resonances of AU7 in *Thermus thermophilus* $tRNA^{Ile}$ which has an identical acceptor sequence (Choi & Redfield, 1986). The ^{15}N shift of this peak, in contrast to its proton shift, is typical for that from a secondary base pair, which tends to rule out the possibility of an unusual mechanism for this proton shift. This unusually shifted resonance was first identified by Hare et al. (1985) and discussed by them. We do not find the unusual temperature lability for this resonance which they mention, at least as compared to the other acceptor AU resonances. The upfield shift of about 1 ppm from the average AU imino proton resonance would be hard to explain by ring-current effects. It could be due to tension in this base pair produced by its connection to the D-stem. The AU7 proton could be stabilized from solvent exchange by the tertiary structure in this region.

The resonances of AU29, GU50, T54–A58, and Ψ55N3H show no noticeable decrease in intensity up to 39 °C, and therefore we conclude that their proton solvent exchange rate is considerably less than 60 s^{-1} up to 39 °C, for the reasons mentioned above. This rate is only a lower limit for the opening rate of the base pair giving the resonance, since the rate-determining step for proton exchange is most likely proton exchange from an unpaired or partly unpaired base. Estimation of opening rates requires measurement of T_2 versus buffer concentration (LeRoy et al., 1985a,b). Nevertheless, rates measured at low buffer concentration might be taken as indicative of stability of base pairs.

Five of the uracil resonance intensities show a definite temperature dependence, as indicated for all but AU6 in Figure 1. Three of these have similar dependence and arise from three acceptor stem resonances, AU4, 6, and 7. The similarity of their temperature dependence suggests that the acceptor stem melts early and possibly in a fairly cooperative manner. A similar conclusion was reached previously for yeast $tRNA^{Phe}$ (Roy & Redfield, 1983).

The temperature dependence of the AU12 resonance intensity is surprising because the D-stem, where this base pair is located, is generally regarded as an especially stable part of the molecule. This supposition is supported by the finding that in yeast $tRNA^{Phe}$, which has an identical D-stem and only has differences in some flanking residues, the GC11 imino proton is stable against exchange for hours, in the presence of magnesium (Figueora et al., 1983). In *E. coli* $tRNA^{Phe}$, which also has the same primary structure as $tRNA^{Val}$ in the D-stem, Hyde and Reid (1985) find a local conformational change at AU12 as indicated by the observation two NMR signals from this base pair. Such a change, occurring at a millisecond rate in $tRNA^{Val}$, could be responsible for the low intensity of our AU12 peak and its temperature dependence. The resonance pattern of the AU12 base pair, and of A9 which forms a triple with it, have been studied previously in yeast $tRNA^{Phe}$ (Choi & Redfield, 1985) as well as in an in vitro synthesized $tRNA^{Phe}$ sequence (Hall et al., 1989).

The lack of temperature dependence of the intensities of anticodon and T stem marker resonances, those of AU29 and of GU50, indicates that these stems are more stable than the acceptor stem. Likewise, the high-temperature persistence of the tertiary marker resonances, T54–A58 and Ψ55, indicate that the TΨC loop and its associated hydrogen bonds to the T loop are also more stable than is the acceptor stem. This result is in contrast to the earliest speculations that tertiary interactions in tRNA's would be least stable and also differs from conclusions from previous early NMR work on yeast $tRNA^{Phe}$ (Johnston & Redfield, 1981b) in which the acceptor stem was proposed to melt at about the same temperature as the tertiary structure.

We did not have enough material and time to investigate these thermal effects more thoroughly. It would be feasible to combine real-time exchange measurements (Figueroa et al., 1983; Choi & Redfield, 1986) with HMQC measurement, as is now routinely done with ^{15}N-labeled proteins (Griffey & Redfield, 1987). Relaxation measurements on individual protons or nitrogens would also be feasible, by edited proton NMR (Redfield et al., 1986), by direct ^{15}N NMR, analogous to measurements on ^{13}C-labeled tRNA (Schmidt et al., 1987), or by heteronuclear 2D methods (Peng et al., 1991). In all cases, it would be desirable to study the buffer dependence of the relaxation rates, in order to separate buffer catalysis

kinetics from helix or tertiary opening rates (Leroy et al., 1985a,b, 1986).

Our experience with fully ^{15}N-labeled tRNA's suggests that extension of these measurements to GC base pairs will be of limited use because the ^{15}N dispersion of GC base pair resonances is small (Redfield et al., 1986). Resonances of GC pairs in unlabeled tRNA's are fairly well separated from those of AU pairs with a few exceptions such as AU12 in the present case and GC11 in yeast $tRNA^{Phe}$ (Heerschap et al., 1982, 1983; Roy & Redfield, 1983). Proton exchange rates in unlabeled macromolecules can also be resolved by combining two-dimensional methods such as NOESY with inversion–recovery sequences (Wüthrich, 1986).

The methodology described in the present paper represents a simple step beyond older one-dimensional methods previously used for RNA's. The synthesis of the precursor uracil is simple, and growth and NMR methods are standard, making the measurements attractive even when more sophisticated methodology is utilized for identification of resonances.

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