15N-labeled Escherichia coli tRNA^Met, tRNA^Glu, tRNA^Tyr, and tRNA^Phe

DOUBLE RESONANCE AND TWO-DIMENSIONAL NMR OF N1-LABELED PSEUDOURIDINE*

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The N1 imino units in Escherichia coli tRNA^Met, tRNA^Glu, tRNA^Phe, and tRNA^Tyr were studied by 1H-15N NMR using three different techniques to suppress signals of protons not attached to 15N. Two of the procedures, Fourier internuclear difference spectroscopy and two-dimensional forbidden echo spectroscopy permitted 1H and 15N chemical shifts to be measured simultaneously at 1H sensitivity. The tRNAs were labeled by incorporation of the uracil autophor S187 on a minimal medium containing [1-15N]uracil. 1H and 15N resonances were detected for all of the N1 imino units except $\psi$13 at the end of the dihydrouridine stem in tRNA^Glu. Chemical shifts for imino units in the tRNAs were compared with "intrinsic" values in model RNAs. Chemical shifts for imino units in the diester backbone may also bind to ribosomes during translation. These interactions permit N1 imino units to stabilize the tertiary structure of a tRNA beyond what is provided by the U it replaces.

Pseudouridine ($\psi$) is a unique C-nucleoside produced by post-translational modification of selected uridines (U) in the tRNAs of prokaryotes and eukaryotes (1). The base is a highly conserved feature of the TΨC sequence at positions 54-56 and frequently occurs in position 39 at the base of the anticodon stem. $\psi$ occurs less frequently at other locations. The role of $\psi$ in tRNAs is not clear. The highly conserved $\psi$53 in the TΨC loop occurs at a sharp bend in the phosphodiester backbone and may also bind to ribosomes during protein biosynthesis (2). $\psi$39 appears to be an essential feature in tRNAs that participate in regulation of the operon for their cognate amino acids (3, 4). Prominent examples are...

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tained 10 pg/ml of [l-15N]uracil. Crude tRNA was obtained by phenol extraction and isopropyl alcohol precipitation. Pure samples of N1-labeled E. coli tRNA, labeled E. coli tRNA, and tRNA were obtained by chromatography on two DEAE-Sephadex columns, the first at pH 7.5 and the second at pH 4.0, according to published procedures (13, 14). The level of incorporation was greater than 90% as determined by the lack of a H peak for unlabeled tRNA between the protons at N1 of 15N and N3 of 15N in tRNA.<ref>There were reported errors in the 15N NMR study of the pseudouridine N1-H units for the four Escherichia coli tRNAs shown in Fig. 1.</ref>

EXPERIMENTAL PROCEDURES

Materials—5'-O-Acetyl-2',3'-O-isopropylidene adenosine was purchased from Sigma. [1-15N]Uracil was synthesized from uracil 15N by the method of Roberts and Poulter (12). A uracil auxotroph of E. coli lacking cytidine deaminase (S6 187) was grown to late log phase at 37 °C on a minimal medium which contained 10 µg/ml of [1-15N]uracil. Crude tRNA was obtained by phenol extraction and isopropyl alcohol precipitation. Pure samples of N1-labeled E. coli tRNA, tRNA, and tRNA were obtained by chromatography on two DEAE-Sephadex columns, the first at pH 7.5 and the second at pH 4.0, according to published procedures (13, 14). The level of incorporation was greater than 90% as determined by the lack of a H peak for unlabeled tRNA between the protons at N1 of 15N in tRNA. 15N NMR (CDC13) 159.0 (N3) and 128.5 ppm (N1).

Preparation of NMR Samples—Samples of tRNA 15N (7.0 mg), tRNA 15N (6.5 mg), and tRNA 15N (6.0 mg) were dissolved in 400 µl of 10 mM cacodylate buffer pH 7.0, which contained 50 mM sodium chloride, 10 mM magnesium chloride, 1 mM EDTA, and 8% deuterium oxide. tRNA 15N (6.0 mg) was dissolved in 400 µl of 100 mM cacodylate buffer pH 7.0, which contained 100 mM sodium chloride, 15 mM magnesium chloride, 1 mM EDTA, and 8% deuterium oxide.

Measurements—H and 15N spectra for the model studies were recorded on a Varian FT80-A NMR spectrometer. 1H chemical shifts were referenced to internal tetramethylsilane or 2,2-dimethyl-2-silapentane-5-sulfonate and 15N chemical shifts, to an external 2.9 M solution of ammonium chloride in 1 M hydrochloric acid. The 15N shifts are given relative to ammonia at 25 °C using a correction factor of 24.9 ppm (17) as previously described (18). NMR data for the tRNAs were obtained on a Nicolet NT 360 MHz spectrometer with a probe triple tuned for 1H, 1H, and 15N and an NTC-1180 data processor (19). The 360 MHz 1H decoupler frequency was mixed down to 36.49 MHz, filtered, amplified, and used for 15N pulses and decoupling. 15N decoupling utilized a WALTZ-16 sequence (20) and the 15N phase variations were controlled by an AdNic Products (Ft. Collins, CO) Black Box.

Three different procedures were used to obtain NMR spectra. Two of the methods, fourier internuclear difference spectroscopy (FINDS) (21, 22) and forbidden echo spectroscopy (FES) (18) yield 1H and 15N chemical shifts for 15N-H units. The third, J-modulated internuclear difference spectroscopy (JIDS), gives 1H resonances for only those protons bound to 15N. We have previously described applications of FINDS and FES to 15N labeled tRNA (18, 21). Application of JIDS is described under "Results."

Two-dimensional data sets consisting of 32 blocks of 1600 transients were collected for tRNA 15N and 15N 15N decays, one with 15N decoupling on-resonance and the other with 15N decoupling off-resonance, are collected, subtracted, and transformed, the 1H signal for the attached proton collapses to a single peak while signals for all other protons in the sample do not change. When two free induction decays, one with 15N decoupling on-resonance and the other with 15N decoupling off-resonance, are collected, subtracted, and transformed, the 1H difference spectrum only shows signals for protons attached to 15N. All other 1H signals disappear. The resulting FINDS signals appear as 3-line derivative patterns with outer lines (the 1H-15N doublet) opposite a signal to the inner line (the decoupled 1H singlet). 15N chemical shifts are obtained by reducing the decoupling power to the minimum needed to collapse the 1H-15N doublet in a decoupling experiment at a single frequency. A series of 1H spectra are obtained as the frequency of the 15N decoupler is varied in small increments. The resonance frequency for the nitrogen in an 1H-15N pair is chosen as the decoupler frequency.


The line width of a FINDS pattern is equal to the sum of the peaks since overlap results in a subtraction that reduces intensity. Some losses are experienced for tRNAs where the conjunction with broad band 15N decoupling, can be used to determine 15N chemical shifts. The 'H doublet centered at 11.7 ppm is attached to a nitrogen whose resonance frequency is outside of the range used for 15N decoupling. Hence, this 'H signal does not change as the 15N decoupler frequency is varied nor does a 'H signal appear at that position in the FINDS spectrum.

FINDS is the most sensitive of the procedures, and in conjunction with broad band 15N decoupling, can be used to scan for 'H-15N units. Sensitivity in a FINDS experiment is a function of the separation of the inner peak and the outer peaks since overlap results in a subtraction that reduces intensity. Some losses are experienced for tRNAs where the proton line widths are from 20 to 35 percent of the 'H-15N coupling constants. Resolution in the 'H dimension is a function of the magnitude of the 'H-15N coupling constant. The line width of a FINDS pattern is equal to the sum of the proton line width plus the 'H-15N coupling constant. tRNAs typically give FINDS patterns 125 Hz wide, and it is sometimes difficult to disentangle signals for 'H-15N units whose 'H and 15N signals are closely spaced. This can limit the accuracy of 15N chemical shifts determined by FINDS. Resolution in the nitrogen dimension is also limited by the level of decoupling power required to collapse the proton doublet, which in some cases may exceed the true nitrogen line width.

Multiple quantum two-dimensional NMR spectroscopy offers several advantages for studying 'H-15N units. Although not as sensitive as FINDS, the two-dimensional FES experiment is an extraordinarily efficient way to determine 15N chemical shifts. A large range of 15N chemical shifts can be measured in a single experiment, and the resolution is only limited by the true proton and nitrogen line widths. The two-dimensional maps for the four tRNAs required less than 6 h of acquisition time with samples approximately 0.5 mM in 15N. Factors that contribute to the high sensitivity include detection of 15N through the higher γ 'H nucleus, elimination of 'H-15N coupling in the 'H signals, and the short duration of the pulse sequence which avoids excessive loss of magnetization from rapid relaxation of the protons. Additional advantages include the chemical shift dispersion inherent in two-dimensional maps and increased reliability for defining 15N chemical shifts (18).

We have used a third procedure, J-modulated internuclear difference spectroscopy (JIDS), to rapidly observe only those protons attached to 15N in a tRNA when nitrogen chemical shift information isn't required. The pulse sequence is shown in Fig. 3. The technique is similar to a classic spin echo experiment with on- and off-resonance 180° pulses on the heteroatom, except the proton 180° pulse is deleted. A selective proton pulse, such as a Redfield 2-1-4 sequence, is used to excite the region of interest without perturbing the large water signal. The spins are allowed to precess for a period Δ1 = 1/2J. During this time, the protons bonded to 12C or 14N are labeled with the frequency of their chemical shift, while protons bonded to 15N are labeled with both their chemical shift and ±1J, depending on the spin state of the nitrogen. When a nitrogen pulse of 180° is applied, the spin state of the nitrogen which the proton sees is interchanged. After an additional time Δ2 = Δ1/2J, the J-modulated frequency information is lost, and the protons bonded to 15N carry no label from the nitrogen. However, if the 180° pulse is not applied, only the protons attached to 15N continue to dephase by a total of π radians, or 180°, during the evolution period of 2(1/2J). When the two experiments are subtracted, the signals from protons bonded to 12C or 14N, which are not frequency modulated by the 180° pulse on 15N, are canceled, and only the signals from protons bonded to 15N remain. The phase error introduced by the effect of the proton chemical shift during the evolution period is resolved by application of a

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**Fig. 2.** Fourier internuclear difference spectroscopy (FINDS) and 'H spectra of [1,3-15N]5-(2',5',8'-trioxadecyl)uracil. A 1:1 mixture of [1,3-15N]5-(2',5',8'-trioxadecyl)uracil and 2',3'-O-isopropylidene-5'-O-acetyl adenosine in chloroform-d at 25 °C, 0.2 mM total nucleoside concentration. FINDS is shown in part a and normal 'H spectra in part b. Sixteen scans were acquired with irradiation of 14N at the chemical shifts indicated between the FINDS and 'H spectra (on-resonance) or at 900 Hz downfield from the indicated chemical shift (off-resonance).

**Fig. 3.** Pulse sequence for J-modulated internuclear difference spectroscopy.
first-order phase correction. This procedure gives a simple singlet for each $^1H$-$^{15}N$ unit, rather than the broad three-line pattern obtained with difference decoupling at high power. The sequence also avoids the 180° pulse on the protons used in spin echo difference spectroscopy, which is difficult to realize in experiments conducted in H$_2$O without overloading the dynamic range of the spectrometer. Application of JIDS to E. coli tRNA$^{\text{Phe}}$ will be presented later in this section.

Model Studies—Formation of hydrogen bonds to the imino protons in nucleic acids is accompanied by downfield shifts in the NMR signals of the proton and the nitrogen. When nucleosides are dissolved in chloroform, the imino protons (23) and nitrogens (24, 25) also experience downfield shifts of similar magnitudes when hydrogen bond acceptors are added to the solution. Model studies with chloroform-soluble derivatives have provided valuable guidelines for assigning NMR peaks of related structures in nucleic acids. Since model shifts were not available for $\Psi$, we initiated a study to determine the intrinsic and hydrogen bonded chemical shifts for the two imino units in the C-nucleoside. 2',3',5'-Tri-O-acetyl pseudouridine was used to determine proton chemical shifts, but because $^{15}N$ labeled $\Psi$ was not available, $^{15}N$ shifts could only be obtained over a very limited range of concentrations by direct observation on an FT-80A NMR spectrometer. A more complete study was conducted with [1,3-$^{15}$N]5-(2',5',8'-trioxo-adecyl)uracil, a chloroform-soluble analog we prepared from [1,3-$^{15}$N]uracil.

The $^1H$ chemical shifts for the protons at N1 and N3 in monomeric $\Psi$ were determined by successive dilution of a chloroform solution of the tri-O-acetyl derivative. The signal for the proton at N1 was assigned by its coupling to the proton at C6. Although splitting could not be clearly discerned at ambient temperature, the peak for N1 was broader due to coupling than that for N3. Below 0 °C, the peak for N1 sharpened into a doublet due to the coupling to the proton at C6 ($J = 2$ Hz). In a 0.2 m chloroform solution of tri-O-acetyl pseudouridine at 26 °C, both imino protons resonated at 10.41 ppm. Dilution of the solution disrupted dimer formation and forced the equilibrium population toward the monomeric state. Signals for the protons at N1 and N3 moved upfield to limiting values at infinite dilution of 8.5 and 8.8 ppm, respectively. These chemical shifts are representative of what one might expect when $\Psi$ is located in a hydrophobic pocket. $^{15}N$ chemical shifts could not be determined at natural abundance with the dilute samples.

Addition of 2',3'-O-isopropylidene-5'-O-acetyl adenosine to a solution of tri-O-acetyl pseudouridine at 26 °C with total nucleoside concentration maintained at 0.2 m produced a downfield shift in the imino protons at N1 and N3 to limiting values of 11.9 and 13.2 ppm, respectively, as shown in Fig. 4. Both resonances moved further upfield to respective values of 13.17 and 14.17 ppm when the sample was cooled to -35 °C. This behavior is similar to that seen for chloroform-soluble derivatives U or T in the presence of A and suggests that both protons in $\Psi$ form hydrogen bonds to A in the model system. The limiting value of 14.17 ppm for the imino proton at N3 in an A$\Psi$ pair is very close to chemical shifts of 14.35 and 14.25 ppm for UA and TA solutions at -35 °C (see Table I). The resonance for the imino proton of G in a GC model is therefore, that one cannot distinguish among imino protons

![Figure 4: $^1H$ and $^{15}N$ chemical shifts for solutions of 2',3',5'-tri-O-acetyl pseudouridine and 5'-acetyl-2',3'-O-isopropylidene adenosine. $^1H$ chemical shifts (□) were measured with solutions 0.2 M in total nucleoside at 26 °C. $^{15}N$ chemical shifts (●) were measured with a 1:1 mixture of 0.5 M in total nucleoside at 26 °C. Part a, N1 imino unit; part b, N3 imino unit.

TABLE I

<table>
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<tr>
<th>Nucleoside</th>
<th>Imino nitrogen</th>
<th>$^1H$ chemical shift</th>
<th>$^{15}N$ chemical shift</th>
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</thead>
<tbody>
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<td></td>
<td></td>
<td>Py r Py r A</td>
<td>Py r Py r A</td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene-5'-O-acetyluridine</td>
<td>N3 8.0° 14.35°</td>
<td>157.3° 163.9°</td>
<td></td>
</tr>
<tr>
<td>2',3'-O-Isopropylidene-5'-O-acetyluridine</td>
<td>N3 8.0° 14.35°</td>
<td>157.3° 163.9°</td>
<td></td>
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<tr>
<td>2',3'-Tri-O-acetyl ribothymidine</td>
<td>N3 8.5° 13.37°</td>
<td>130.2°</td>
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<tr>
<td>2',3'-Tri-O-acetyl pseudouridine</td>
<td>N3 8.5° 13.37°</td>
<td>130.2°</td>
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</tr>
<tr>
<td>5'(2',5',8'-Trioxo-adecyl)uracil</td>
<td>N3 13.0° 161.5°</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

Chloroform solution at 26 °C extrapolated to infinite dilution (26).

Chloroform solution, 0.02 m pyrimidine and 0.18 m 2',3'-O-isopropylidene-5'-O-acetyl adenosine at -35 °C.

Chloroform solution, 0.2 m nucleoside at 26 °C.

Chloroform solution extrapolated to infinite dilution at 26 °C.

Chloroform solution, 0.5 m at 26 °C.

Chloroform solution, 0.02 m pyrimidine and 0.18 m 2',3'-O-isopropylidene-5'-O-acetyl adenosine at 26 °C.
in AU, AV, AT, or GC pairs or between syn and anti AΨ conformers solely on the basis of proton chemical shifts.

\(^{15}\text{N}\) chemical shifts were measured for 0.2 M solutions of tri-O-acetyl pseudouridine and for solutions 0.1 M in tri-O-acetyl pseudouridine and the chloroform-soluble adenosine derivative. The N1 resonance moved downfield from 130.6 to 131.9 ppm, while that for N3 moved from 158.9 to 161.0 ppm. Although we had clearly not attained the concentration of A needed for limiting values, \(^{15}\text{N}\) signals could not be measured at lower concentrations of \(\Psi\). These measurements were, however, sufficient to demonstrate the large difference in nitrogen chemical shifts for N1 and N3 and that both resonances are sensitive to the formation of hydrogen bonds.

An analog of \(\Psi\), [1,3,\(^{15}\text{N}_{2}\)5-(2',5',8'-trioxadecyl)uracil was prepared so the chemical shifts of the pyrimidine nitrogens could be measured over a wider range of concentrations. As shown in Fig. 5, at 26 °C the proton shifts of the N1 and N3 units in the analog moved downfield from 10.2 and 10.4 ppm to 12.0 and 13.3 ppm, respectively, as the mole fraction of adenosine was increased to 0.9. These changes were virtually identical to those observed for tri-O-acetyl pseudouridine and the chloroform-soluble adenosine derivative. The N1 resonance moved downfield from 130.6 to 131.9 ppm, while that for N3 moved from 158.9 to 161.0 ppm.

Although we had clearly not attained the concentration of A needed for limiting values, \(^{15}\text{N}\) signals could not be measured with solutions 0.2 M in total nucleoside at 26 °C. These comparisons of tri-O-acetyl

\[^{1}\text{H}\] and \(^{15}\text{N}\) chemical shifts were measured for 0.2 M solutions of [1,3,\(^{15}\text{N}_{2}\)5-(2',5',8'-trioxadecyl)uracil and 5'-acetyl-2',3'-O-isopropylidene adenosine. \[^{1}\text{H} (\bullet)\] and \(^{15}\text{N} (\circ)\) chemical shifts measured with solutions 0.2 M in total nucleoside at 26 °C. Part a, N1 imino unit; part b, N3 imino unit.

![Fig. 5. \[^{1}\text{H}\] and \(^{15}\text{N}\) chemical shifts for solutions of [1,3,\(^{15}\text{N}_{2}\)5-(2',5',8'-trioxadecyl)uracil and 5'-acetyl-2',3'-O-isopropylidene adenosine. \[^{1}\text{H} (\bullet)\] and \(^{15}\text{N} (\circ)\) chemical shifts measured with solutions 0.2 M in total nucleoside at 26 °C. Part a, N1 imino unit; part b, N3 imino unit.](image)

Fig. 6. A \[^{1}\text{H}-^{15}\text{N}\] FINDS spectrum at 15 °C for \(E\). coli tRNA\(^{33}\) from \(E\). coli grown on \[^{15}\text{N}\]uracil. The sample was dissolved in 10 mM cacodylate buffer, pH 7.0, containing 50 mM sodium chloride, 10 mM magnesium chloride, 1 mM EDTA, and 8% (v/v) deuterium oxide. A total of 2400 scans was accumulated with broadband irradiation of \(^{15}\text{N}\) chemical shift (on-resonance) and 1000-Hz downfield (off-resonance) on alternate scans.

\(|\Psi\) and the analog with uridine in the presence of adenosine are sufficient to demonstrate that one cannot unambiguously distinguish between A\(-U\) and A\(-\Psi\) pairs by \[^{1}\text{H}\] and \(^{15}\text{N}\) chemical shifts when \(\Psi\) is in the anti conformation. An A\(-\Psi\) pair with \(\Psi\) in the syn conformation, however, should have a H\(-N1\) imino unit with a proton chemical shift near 13 ppm correlated with a unique nitrogen resonance near 132 ppm. The results of the model studies are summarized in Table I.

\(^{1}\text{H}\) and \(^{15}\text{N}\) NMR Spectra of N1 Labeled tRNAs—\(E\). coli tRNA\(^{33}\) has a single \(\Psi\) at position 55. Correspondingly, a single \[^{1}\text{H}\] peak at 10.57 ppm was observed in the FINDS and FES spectra shown in Figs. 6 and 7. The FINDS spectrum gave a correlated nitrogen shift of 134.9 ppm. Clearly, access of the N1 imino proton to bulk solvent is impeded, either by steric exclusion of water or a combination of steric exclusion and hydrogen bonding. The proton and nitrogen chemical shifts are in between values expected for a free N1 unit and one paired with A or a phosphate oxygen. The \[^{1}\text{H}\] shift is in the range expected for \(\Psi\)-oxygen interactions when the oxygen donor is a sugar hydroxyl or an immobilized water molecule.

The FES map of tRNA\(^{33}\) at 15 °C shown in Fig. 8 has a single peak with proton and nitrogen shifts of 10.42 and 135.4 ppm. This resonance is very close to the 10.57 and 134.9 ppm peak for \(\Psi\) in the two-dimensional map for tRNA\(^{33}\) and is assigned to the corresponding base in tRNA\(^{33}\). A second peak for \(\Psi\) is not observed. The small peak seen at 10.5 and 141.0 ppm has a nitrogen shift outside of the known range for N1 units and is attributed to noise. We conclude that the N1 proton in \(\Psi\) exchanges rapidly with bulk water and a discreet signal is not seen or the resonances for \(\Psi\) and \(\Psi\) overlap in both dimensions, a situation not observed for other tRNAs with more than one \(\Psi\).

\(E\). coli tRNA\(^{33}\) has \(\Psi\) at positions 39 and 55. A FES spectrum of the molecule shows peaks for two \[^{1}\text{H}-^{15}\text{N}\] units.

\[^{2}\text{D. R. Davis and C. D. Poulter, unpublished results.}

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\[^{\ast}\text{D. R. Davis and C. D. Poulter, unpublished results.}\]
$^{15}$N-labeled *E. coli* Double Resonance and Two-dimensional NMR

$^{1H}$ and $^{15}$N shifts for the $\Psi 93$ N1 unit are again consistent with hydrogen bonding to a neighboring sugar hydroxyl or an immobilized molecule of water. The $^1H$ chemical shift is clearly outside the range expected for an A-Ψ interaction, demonstrating that $\Psi 93$ does not pair with adenosine in a syn A-Ψ Watson-Crick structure under the conditions of the NMR measurement.

*E. coli* tRNA$^{\text{Phe}}$ has pseudouridines located at positions 32, 39, and 55. A JIDS spectrum (see Fig. 9) has $^1H$ peaks at 10.45 and 10.60 ppm with the latter peak having approximately twice the intensity of the former. No $^1H$ signal is observed between 12.5 and 14.0 ppm in the region where the imino proton in an AΨ pair in the syn conformation would be expected. The enhanced dispersion provided by $^1H$-$^{15}$N chemical shift correlation is nicely illustrated by the FES map shown in Fig. 10 where three well-resolved peaks at 10.45/132.9, 10.60/135.3, and 10.65/132.5 ppm are seen. The two-dimensional resonance at 10.60/135.3 ppm has $^1H$ and $^{15}$N shifts which most nearly match those assigned to $\Psi 55$ in tRNA$^{\text{Tyr}}$ and tRNA$^{\text{Thr}}$. The tandem shifts to lower field for tRNA$^{\text{Tyr}}$ indicate a slight change in the environment of the N1 unit, perhaps reflecting a conformation change in the tWC loop. The peaks at 10.45/132.9, and 10.65/132.5 ppm have chemical shifts similar to that observed for $\Psi 39$ in tRNA$^{\text{Tyr}}$ and suggest that the structure of the A$^{31}$-Ψ$^{39}$ pair is similar in both tRNAs. One of these two signals must come from $\Psi 32$ in the anticodon loop. Chemical shifts for the tRNAs are summarized in Table II.

**DISCUSSION**

One of the major limitations of NMR for the study of biopolymers is the high density of peaks in the spectrum. Regions with closely spaced peaks are difficult or impossible to interpret because the resonances cannot be resolved and assigned to specific structural features. This situation is further exacerbated by the broader peaks usually found when studying larger molecules. The power of $^1H$-$^{15}$N chemical shift correlation in conjunction with site-specific labeling for circumventing problems associated with spectral density is evident. Of the more than 900 protons and 250 nitrogens in a
When we began this project there were only a few assignments for \( \Psi \) imino protons and no nitrogen measurements in the literature. Tropp and Redfield (27) had attributed a resonance at 10.60 ppm in \( E.\ coli \) tRNA\(^{\text{met}} \) to the imino proton at N1 of \( \Psi 55 \) on the basis of an elegant experiment in which they observed an NOE from the methyl group in T54. They further proposed that the N1 proton forms a hydrogen bond to a water molecule in the interior of the TWC loop. We examined this region in the x-ray crystal structure of \( E.\ coli \) tRNA\(^{\text{met}} \) and found two possible interactions (28). One is to a water molecule positioned between the N1 proton and P54, and the other, to the 2'-sugar hydroxyl in T54. Either possibility can be accommodated by a small conformational change in \( \Psi \). Our data do not permit us to distinguish between the two possibilities. \( \Psi 55 \) is located at a sharp bend in the phosphodiester backbone, and the N1 hydrogen bond may help to stabilize a region under stress.

The conformation of \( \Psi \) in A-\( \Psi \) pairs has been of interest for several years, especially the A31-\( \Psi 39 \) interaction found at the base of the anticodon stem in several regulatory tRNAs. Reid and Hurd (6,7) originally proposed an atypical syn structure for the A31-\( \Psi 39 \) pair in yeast tRNA\(^{\text{met}} \) on the basis of an assignment of the N1 proton resonance to a peak at 13.1 ppm. Roy and co-workers (8,9) also proposed a syn conformation for the pair but recently revised their assignment (11). Their original assignment was based on an NOE from an imino resonance at 13.2 ppm to an aromatic resonance at 6.85 ppm. The signal persisted in a sample deuterated at the purine C2 position. The most likely candidate for the NOE was the proton at C6 of \( \Psi \), hence the assignment of the 13.2 resonance to the adjacent proton at N1. Unfortunately, deuteration was not complete, and the NOE was apparently to the C2 proton of A31 in residual unlabeled purine. Using a two-dimensional method similar to ours, they now assign the N1 and N3 units in \( \Psi 39 \) to signals at 10.6/135 ppm and 13.2/163 ppm, respectively. Our results for \( E.\ coli \) tRNA\(^{\text{met}} \) and tRNA\(^{\text{met}} \) clearly support an anti A31-\( \Psi 39 \) pair for these molecules as well. The N1 protons for \( \Psi 39 \) in the \( E.\ coli \) and yeast tRNAs all resonate in the vicinity of 10.5 ppm, a chemical shift consistent with hydrogen bonding of the imino proton to a water molecule or a sugar hydroxyl. The x-ray structure of yeast tRNA\(^{\text{met}} \), with \( \Psi 39 \) in the anti conformation places N1 approximately 5.5 Å from an oxygen at P38 (29), a distance compatible with hydrogen bonding of the attached proton to a bridging water molecule. There are no nearby sites for a hydrogen bond directly to another part of the tRNA.

Anti A31-\( \Psi 39 \) pairs in yeast tRNA\(^{\text{met}} \), \( E.\ coli \) tRNA\(^{\text{met}} \), and \( E.\ coli \) tRNA\(^{\text{met}} \) have topologies very similar to normal A-U pairs. In all cases the proton at N3 is bound to A. Although the locations of C2 and C4 interchange for U and \( \Psi \), the topology of the pyrimidine moiety is similar when viewed from A. The most significant difference between anti A-\( \Psi \) and A-U pairs is the additional locus for hydrogen bonding present at N1 in \( \Psi \). The small increment in stabilization may be important for reducing conformational mobility in the region of the tRNA which contains \( \Psi \). A-\( \Psi \) pairs often are found at the ends of helices where A-U interactions are susceptible to fraying. It should be noted that we failed to detect a resonance for N1 of \( \Psi 13 \) located at the end of the dihydrouridine stem in \( E.\ coli \) tRNA\(^{\text{met}} \). Perhaps the N1 proton is involved in hydrogen bonding but the interaction is too weak to prevent rapid exchange with water or factors other than stabilization by the N1 proton dictate replacement of U by \( \Psi \) at that position.

It is also noteworthy that we saw a resonance for the N1
proton of $\Psi$32 in the anticodon loop of E. coli tRNA$_{Phe}$ with a chemical shift also in the vicinity of 10.5 ppm. As previously discussed for the N1 imino hydrogens in $\Psi$ at other locations, this value indicates hydrogen bonding to a water molecule or a neighboring sugar hydroxyl. Although no x-ray data are available for E. coli tRNA$_{Phe}$ an interesting observation can be made using the structure for yeast tRNA. C5 of U32 in yeast tRNA$^{Phe}$ is 5-5.5 A from oxygens attached at P31 and P32. This position corresponds to the location of N1 if U32 were converted to pseudouridine and changes in overall topology were minimized. The distances are perfect for a hydrogen bond between the N1 proton of $\Psi$32 and a water molecule bridged to one of the neighboring phosphate oxygens at P31 or P32. Such an interaction would help stabilize the conformation of the anticodon loop. Additional work will be needed to verify this structure for E. coli tRNA$^{Phe}$.

$^1$H-$^15$N chemical shift correlation, especially the two-dimensional forbidden echo technique, is a powerful tool for the study of biopolymers in aqueous solutions. The pulse sequence is relatively straightforward and can be performed on most modern high field NMR spectrometers. The sensitivity and resolution of the techniques presented in this paper are inversely proportional to the proton line width for the imino unit, and ultimately the methods will fail for large biopolymers because of excessive broadening of the resonances. We have, however, recently obtained two-dimensional maps of 5 S RNA where the line widths are approximately twice as wide as the 20-Hz lines typically seen for tRNAs. Of course, the proton in any $^1$H-$^15$N unit must be exchanging slowly with solvent to be observed, which limits detection to bound protons on the surface of a biopolymer or inaccessible protons in the interior of the molecule. Fortunately, the most important protons with respect to structure or catalysis are those which form hydrogen bonds. Although the specific applications discussed in this paper use $^{13}$N-labeled tRNAs, the high sensitivity of FINDS and FES make them ideally suited for obtaining $^{13}$N spectra of protonated nitrogens at natural abundance when quantities of samples are not limited. Other extensions of the techniques include $^1$H-$^{13}$C chemical shift correlation for protonated carbons and metabolic studies with $^{15}$N or $^{13}$C in vivo.

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