^1H–^{15}N NMR Studies of *Escherichia coli* $tRNA^{Phe}$ from *hisT* Mutants: A Structural Role for Pseudouridine†

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ABSTRACT: *Escherichia coli* $tRNA^{Phe}_{U39}$ was isolated from a specially constructed bacterial strain (DD1003/pRK3) carrying mutations in the *hisT* gene (the structural gene for tRNA pseudouridine synthase I) and in the *pyrB* gene (uracil auxotrophy). The *pheU* gene for $tRNA^{Phe}$ under control of the native tRNA promoter was on a multicopy plasmid and gave up to 40-fold overproduction of $tRNA^{Phe}_{U39}$. The double mutant permitted efficient incorporation of [3-^{15}N]uracil, resulting in >95% ^{15}N enrichment of the uracil-derived bases. ^1H and ^1H–^{15}N NMR experiments were used to assign the low-field proton resonances to specific hydrogen-bonding interactions. ^1H NMR assignments indicate that $tRNA^{Phe}_{U39}$ has a structure similar to that of native $tRNA^{Phe}$ except in the anticodon region where replacement of pseudouridine (Ψ) at position 39 with uridine (U) destabilizes hydrogen-bonding interactions at the base of the anticodon stem. We propose that U → Ψ modifications further stabilize interactions normally available to U by providing an additional locus for hydrogen bonding to the pyrimidine ring.

Transfer RNA differs from other nucleic acids in the high percentage of its nucleosides that are modified (Nishimura, 1977). In tRNAs from eubacteria, for example, modified bases constitute approximately 10% of the total, and it is estimated that at least 40 different enzymes, occupying 1% of the coding capacity of the bacterial genome, are required to produce mature tRNA from an unmodified transcript. Modified bases are typically derived from the normal nucleosides adenosine (A), guanosine (G), uridine (U), and cytidine (C) (Björk et al., 1987). In spite of this substantial genetic investment, studies of mutants that lack specific modifications indicate that most are not essential for viability. Recently it was reported that unmodified yeast $tRNA^{Phe}$ (Sampson & Uhlenbeck, 1988) and *Mycoplasm mycoides* $tRNA^{Gly}$ (Samuelson et al., 1988) synthesized in vitro with T7 RNA polymerase are substrates for aminoacylation with kinetics similar to their fully modified counterparts. Furthermore, unmodified $tRNA^{Gly}$ was essentially as efficient as the fully modified tRNA for protein synthesis in vitro. ^1H NMR studies with unmodified yeast $tRNA^{Phe}$ indicate that the molecule folds normally in the presence of high concentrations of Mg^{2+}, although the stability of the structure is substantially reduced relative to the fully modified molecule (Hall et al., 1989). It is clear, however, that base modification plays an important biological role in fine tuning the efficiency of tRNAs during decoding, improving the fidelity of protein biosynthesis, and maintaining the proper reading frame. How this is accomplished is not understood at the molecular level (Nishimura, 1977; Björk et al., 1987; Clark, 1977; Samuelsson & Olsson, 1990).

In terms of distribution and frequency of occurrence, pseudouridine (Ψ)[1] is the most common modified nucleoside. It is typically found in regions where stems join loops and at position 55, the most highly conserved of all tRNA modifications (Nishimura, 1977). Five pseudouridines (Ψ13, Ψ38, Ψ39, Ψ40, and Ψ55) belong to a very small family of modified nucleosides found in cytosolic tRNAs of organisms from all three biological kingdoms (Björk et al., 1987). In addition to tRNAs, Ψ is present in large bacterial and eukaryotic ribosomal RNAs (Fellner & Sanger, 1968; Hughes et al., 1976; Choi & Busch, 1978; Branlant et al., 1981), 5S RNAs from yeast (Rubin, 1973), 5.8S RNA of most eukaryotic ribosomes (Miyazaki, 1974; Erdmann et al., 1983), and several nuclear RNAs (Busch et al., 1982).

A mutation in the *hisT* gene of *Salmonella typhimurium* or *Escherichia coli* (the structural gene for tRNA pseudouridine synthase I) results in undermodification at positions 38–40 (Turnbough et al., 1979; Kammen et al., 1988). The mutants have reduced growth rates, reduced polypeptide chain elongation rates, and increased error levels during translation (Parker, 1982; Palmer et al., 1983). In addition, the histidine operon in these bacteria is derepressed (Roth et al., 1966) by upsetting the transcription attenuation mechanism that depends on how efficiently a leader coding sequence rich in histidine codons is translated (Landick & Yanofsky, 1987). Since these bacteria have several tRNAs with Ψ in positions 38–40 (Sprinzl et al., 1987) and operons for the corresponding amino acids with leaders rich in repetitive codons for these tRNAs (Landick & Yanofsky, 1987), *hisT* mutations probably influence regulation of several biosynthetic pathways.

Ψ is considerably more versatile in its hydrogen-bonding interactions than the U it replaces. For example, the pyrimidine ring in an A-U Watson–Crick pair adopts a conformation with the C(2) carbonyl group anti to the ribose, as shown in Figure 1. Ψ can form a structure whose topology is almost identical by engaging A with its C(2) carbonyl and N(3) imino proton. In this orientation the N(1) imino hydrogen presents an additional locus for hydrogen bonding to the modified nucleoside not available to U. Although Ψ can also interact with A through its C(2) carbonyl and N(1) imino proton in a syn Watson–Crick base pair, NMR studies in our laboratory (Griffey et al., 1985) and by Roy et al. (1984) show this is not the case. A31–Ψ39 pairs at the base of the anticodon stems of yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Phe}$, and *E. coli* $tRNA^{Tyr}$ are anti Watson–Crick structures. In addition, all

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[1] Abbreviations: Ψ, pseudouridine; HMQC, heteronuclear multiquantum correlation; CSA, chemical shift anisotropy; NOE, nuclear Overhauser enhancement.

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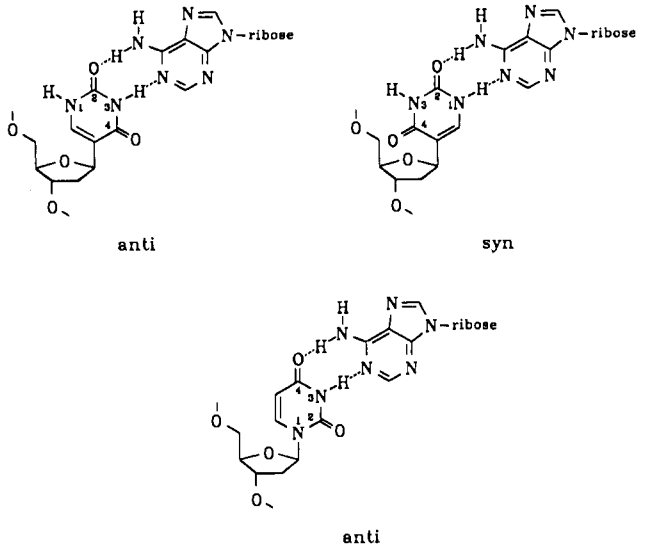

FIGURE 1: Anti AΨ, syn AΨ, and anti A-U Watson–Crick base pairs.

of the N(1) imino protons thus far identified in tRNAs form stable tertiary hydrogen bonds. ^{1}H chemical shifts indicate that the hydrogen-bond acceptors are neutral sp^3-hybridized oxygens like those found in ribose hydroxyl groups or water molecules. In order to assess the effects of modification at position 39, we isolated *E. coli* $tRNA^{Phe}_{U39}$, a selectively undermodified molecule, and now describe the results of NMR experiments with the fully modified and selectively undermodified tRNAs that address the effect of modification on stability at the junction of the anticodon stem and loop.

Materials and Methods

Materials. Nuclease P1, bacterial alkaline phosphatase, and all antibiotics were purchased from Sigma. Benzoylated DEAE-cellulose (BD-cellulose) and ATP were from Boehringer-Mannheim. [U-^{14}C]Phenylalanine was purchased from Amersham. [3-^{15}N]Uracil was obtained as previously described (Griffey & Poulter, 1983). NMR samples were prepared by dissolving 8–40 mg of lyophilized tRNA in 0.4 mL of NMR buffer, consisting of 10 mM cacodylate buffer, 100 mM sodium chloride, 10 mM magnesium chloride, and 0.5 mM EDTA, pH 7.0, in 92:8 (v/v) $H_2O/^2H_2O$.

NMR Spectroscopy. A Varian VXR-500 NMR spectrometer was used for all experiments at 500 MHz. The spectrometer was equipped for dual broad-band operation and had a ^{1}H observe/^{15}N decouple probe. For ^{1}H–^{15}N experiments, bilevel decoupling was employed resulting in a ^{15}N $\pi/2$ pulse of 65 μs and $\gamma H2/2\pi$ = 2500 Hz for decoupling during acquisition. The ^{1}H transmitter power was attenuated under software control to give an approximate $\pi/2$ pulse width of 24–28 μs to optimize the performance of the selective solvent-suppression pulses. The modified "1–2–1" pulse sequence described by Starcuk and Sklenar (1983) was used for proton $\pi/2$ and π pulses where solvent suppression was required. This pulse sequence provided excellent solvent suppression and good phase and amplitude behavior across the region of interest. The ^{1}H transmitter was placed at 12.5 ppm to maximize excitation of the imino protons. A spectral window was chosen so that the H_2O peak was at the extreme upfield edge. The interpulse delay was approximately $1/2\Delta\omega$ and was adjusted for maximal suppression; the τ delay between the components of the "2" pulse was 10 μs.

HMQC spectra were obtained without the ^{1}H π pulse commonly used in similar experiments on small molecules; pulse and receiver phases were cycled to select for the ^{1}H–^{15}N zero-quantum frequencies (Bax et al., 1983). The ^{1}H π pulse was omitted due to the favorable relaxation of the ^{1}H–^{15}N zero-quantum coherences, which give a spectrum with greater sensitivity than the refocussed experiment (Griffey & Redfield, 1987). 2D Fourier transformation of these data resulted in spectra with $(F_1,F_2) = (\delta_H + \delta_N,\delta_H)$. The ^{15}N chemical shifts were measured from the ^{15}N carrier frequency, a line of slope = 1 through the F_1,F_2 zero-frequency point in the center of the 2D plot (Bax et al., 1983; Griffey et al., 1983b). ^{1}H chemical shifts for tRNA were referenced to DSS. ^{15}N chemical shifts were referenced to the frequency of a saturated ammonium nitrate solution at 20.68 ppm (Levy & Lichter, 1979).

Strain Constructions. LB was used for a rich medium and M9 was used for a minimal medium (Maniatis et al., 1982). Auxotrophic requirements were satisfied by supplementing minimal media with 10 μg/mL uracil. *E. coli* strains MA1006(*pyrB*$^-$), GR401(*pyrB*$^+$::*tn10*), and FB105(*hisT*$^-$) were obtained from the *E. coli* Genetic Stock Center at Yale University. P1 phage mediated transductions were as described by Curtiss (1981); all other manipulations were according to Maniatis et al. (1982). DD1003 and MA1006 were transformed with pRK3 according to the calcium chloride procedure. Fully modified *E. coli* $tRNA^{Phe}$ was isolated from 10-L incubations of MA1006/pRK3 (*amp*^{R}, *pyrB*$^-$, *pheU*), and undermodified material was obtained from DD1003/pRK3 (*amp*^{R}, *tn10*::*pyrB*$^-$, *hisT*$^-$, *pheU*).

Isolation and Analysis of tRNA. *E. coli* strains MA1006/pRK3 and DD1003/pRK3 were incubated at 37 °C in a 10-L fermentor (New Brunswick Microgen), and growth was monitored by measuring the optical density at 600 nm. Labeled tRNA was obtained from incubations in a minimal medium containing [3-^{15}N]uracil. A soluble RNA fraction was obtained from wet cells by phenol extraction followed by ethanol precipitation. The material was chromatographed on BD-cellulose, pH 6.0, by using a linear gradient of 0.4–1.5 M sodium chloride (Gillam et al., 1965). The last major UV-active peak to elute contained $tRNA^{Phe}$ as determined by aminoacylation with *E. coli* $tRNA^{Phe}$ synthetase (Louie et al., 1984). The purified $tRNA^{Phe}$ or $tRNA^{Phe}_{U39}$ accepted 1800–1900 pmol of phenylalanine per A_{260} unit of tRNA and gave a single peak by HPLC on a C4 reversed-phase column (Pearson et al., 1983).

Nucleoside Analysis. The pattern and extent of modification for $tRNA^{Phe}$ and $tRNA^{Phe}_{U39}$ was determined by HPLC. Samples for HPLC were prepared by digesting approximately 125 μg of pure isoacceptor with nuclease P1 and dephosphorylating the nucleotides with alkaline phosphatase (Gehrke et al., 1983). The mixtures of nucleosides were analyzed by gradient HPLC on a C18 reversed-phase column (Buck et al., 1983).

Results

Construction of MA1006/pRK3 and DD1003/pRK3 and Production of Labeled E. coli $tRNA^{Phe}$ and $tRNA^{Phe}_{U39}$. Fully modified *E. coli* $tRNA^{Phe}$ was isolated from stationary phase cultures of MA1006/pRK3 (*amp*^{R}, *pyrB*$^-$, *pheU*) obtained by transforming MA1006 with pRK3, a high copy number plasmid containing an *E. coli* gene encoding $tRNA^{Phe}$ under control of its natural promoter (Schwartz et al., 1983). In preliminary runs, samples were removed periodically, and tRNAs were analyzed by HPLC of phenol-extracted material. We found that modification was normally complete after the cultures had been in stationary phase for 2 h. A typical 10-L fermentation at 37 °C in M9 medium supplemented with 100 mg of uracil yielded 15–20 mg of purified isoaccepting

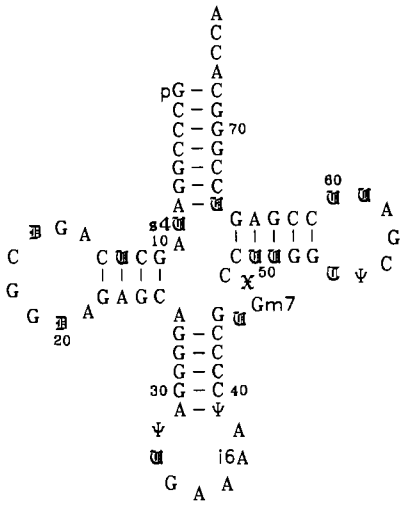

FIGURE 2: Cloverleaf structure of $tRNA^{Phe}$. The uridine-related bases labeled with ^{15}N are in script. In $tRNA^{Phe}_{U39}$ the pseudouridine at position 39 has been replaced with an unmodified uridine.

$tRNA^{Phe}$. This material gave a single peak on HPLC.

Incubation of MA1006/pRK3 in M9 supplemented with [3-^{15}N]uracil produced $tRNA^{Phe}$ selectively labeled at N(3) of its pyrimidine bases. The location and extent of labeling was confirmed by NMR (see below) and mass spectrometry. Labeled tRNA was digested, and labeled uridine was isolated by HPLC under the same conditions as used for nucleoside analysis. The uridine was analyzed by mass spectrometry (Pang et al., 1982). The peak at m/z = 517 corresponding to the molecular ion for uridine was compared to the peak at m/z = 518 for ^{15}N uridine and indicated an isotopic enrichment of greater than 95%.

E. coli $tRNA^{Phe}_{U39}$, selectively undermodified at position 39, was obtained from DD1003/pRK3 (*amp*^{R}, *pyrB*$^-$::*tn10*, *hisT*$^-$, *pheU*) as described for fully modified $tRNA^{Phe}$ (see Figure 2). The host strain DD1003 for pRK3 was constructed by phage P1 mediated transduction according to the strategy outlined in Figure 3. The initial step linked a *tn10* tetracycline marker to the *pyrB*$^-$ gene by transducing MA1006 (*pyrB*$^-$) with phage grown on GR401 (*pyrB*$^+$::*tn10*), selecting for tetracycline resistance, and screening for uracil auxotrophy. Phage grown on a resulting strain, DD1001, was then used to transduce FB105 (*hisT*$^-$), and colonies were again selected for tetracycline resistance and screened for uracil auxotrophy to yield DD1003, which was then transformed with pRK3. When colonies from the transduction requiring uracil were scored against 96 tetracycline-resistant colonies, the co-transductional frequency for *tn10* and *pyrB*$^-$ was 5%.

DD1003/pRK3 produced $tRNA^{Phe}_{U39}$ lacking the single modification U39 → Ψ39 (Kammen et al., 1988). $tRNA^{Phe}_{U39}$ was obtained as described for $tRNA^{Phe}$, and loss of only one of the three pseudouridines was verified by HPLC. Mixtures of nucleosides obtained by digestion of separate samples of $tRNA^{Phe}$ and $tRNA^{Phe}_{U39}$ with nuclease P1 and treatment of the resulting nucleotides with alkaline phosphatase were analyzed by HPLC on a C18 column (Buck et al., 1983; Gehrke et al., 1983). As shown in Figure 4, the intensity of the pseudouridine peak in the hydrolysate from $tRNA^{Phe}_{U39}$ was 30% lower than for hydrolysate from fully modified $tRNA^{Phe}$. The DD1003/pRK3 system also gave high levels (>95%) of selectively labeled tRNA when grown in M9 supplemented with [3-^{15}N]uracil.

^{1}H–^{15}N NMR Experiments. An ^{1}H–^{15}N difference decoupling experiment was initially used to selectively observe only those protons attached to ^{15}N in labeled $tRNA^{Phe}_{U39}$,

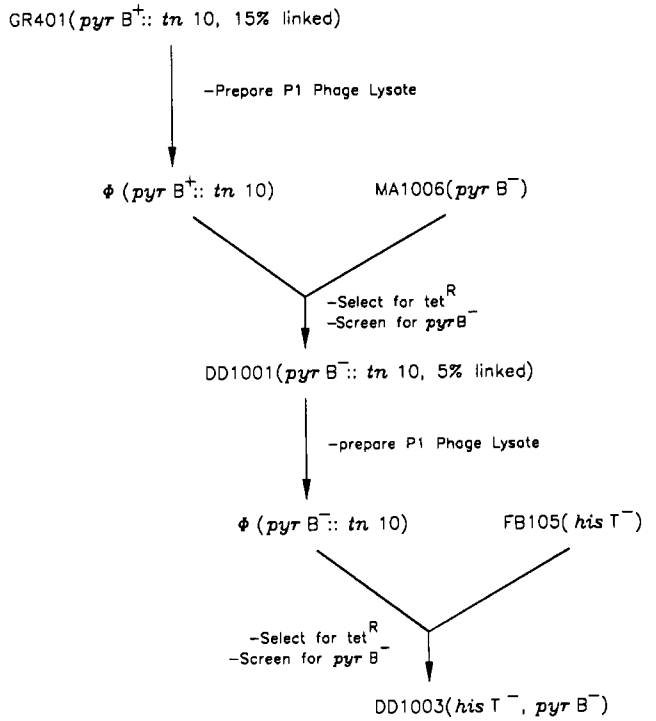

FIGURE 3: Construction of the *E. coli* double mutant DD1003 containing the *pyrB*$^-$ gene conferring uracil auxotrophy and the *hisT*$^-$ gene disabling the modification of U39 → Ψ39 in $tRNA^{Phe}$.

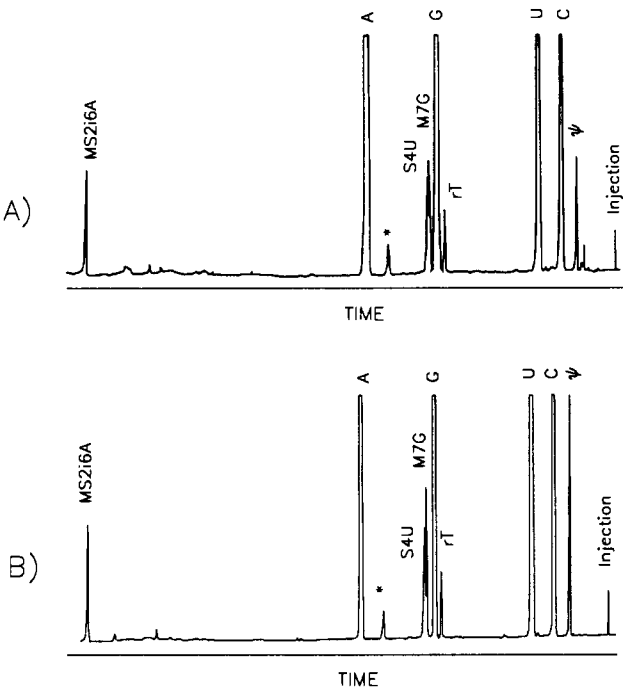

FIGURE 4: HPLC chromatographic traces from the nucleoside analysis of (A) $tRNA^{Phe}_{U39}$ and (B) $tRNA^{Phe}$. Note the reduction of the Ψ peak for the undermodified tRNA compared to the native material. Quantitation of the peaks indicated that the pseudouridine peak was reduced by approximately 30% in (A). Approximately 10 μg of the digested tRNA isoacceptor was injected for each analysis. The column temperature was 30 °C, and a simple linear gradient was employed during elution. The retention time of all major modified nucleosides was verified by injection of standard samples.

thereby eliminating the guanosine imino protons from the spectrum (Griffey et al., 1985). Figure 5 shows ^{1}H–^{15}N difference decoupling spectra for labeled $tRNA^{Phe}_{U39}$ at 15 and 21 °C. This spectrum contains five distinct trio patterns, indicating proton exchange for the resolved uridine resonances is still slow on the NMR time scale at 21 °C. Preliminary

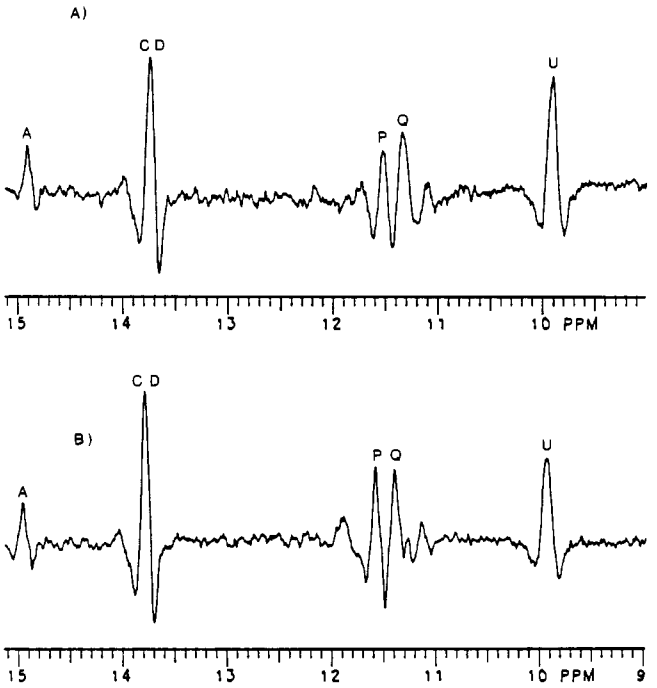

FIGURE 5: $^1H-^{15}N$ double resonance difference decoupling spectra of [3-^{15}N]uracil-labeled $tRNA^{Phe}_{U39}$. A total of 1500 off-resonance transients were subtracted from 1500 on-resonance transients to provide the difference spectrum. (A) Spectrum at 15 °C; (B) spectrum at 21 °C.

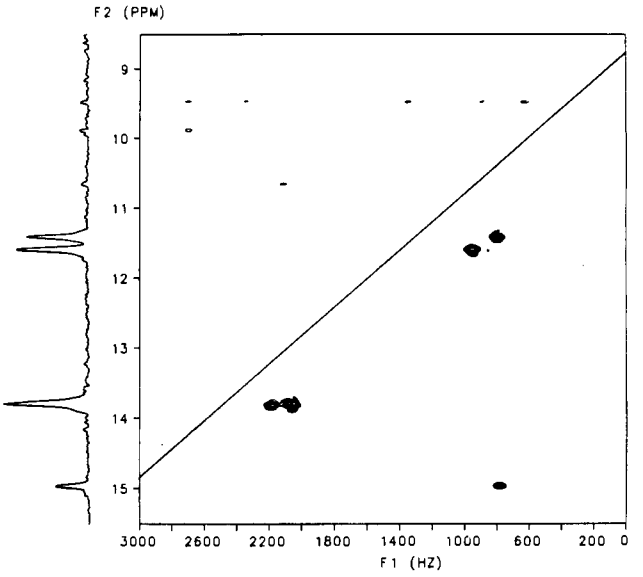

FIGURE 6: $^1H-^{15}N$ 2D HMQC spectrum of [3-^{15}N]uracil-labeled $tRNA^{Phe}_{U39}$ at 26 °C. This experiment results in a 2D plot of ^1H vs $^1H-^{15}N$ multiple-quantum frequency. The modified "1–2–1" pulse was used for the selective ^1H 90° pulse. A total of 2500 transients were acquired for each t_1 increment into 2048 data points. The t_1 dimension was zero filled from 96 to 256 points prior to transformation. The spectrum is presented in absolute value mode.

assignments for the five resolved peaks were based on previous work with $tRNA^{Phe}$ (Hyde & Reid, 1985). A peak at 14.87 ppm was assigned to s^{4}U8-A14 because of its downfield ^1H chemical shift (Hurd & Reid, 1979). The intense peak at 13.75 ppm is probably a composite of the three Watson–Crick secondary A-U's and the T54–A58 tertiary interaction in $tRNA^{Phe}_{U39}$ (Hyde & Reid, 1985). The lack of resolution is a result of coincident chemical shifts and the broad (ca. 180 Hz) trio patterns for each resonance inherent in the $^1H-^{15}N$ difference decoupling experiment with imino protons. Peaks at 11.55 and 11.33 ppm were assigned to Ψ55(N3-H) and U51-G63, respectively, in analogy with assignments for fully modified $tRNA^{Phe}$ (Hyde & Reid, 1985). A resonance at 9.88 ppm was attributed to D16 and D20 (Davis et al., 1986). The intensity of the peak suggests that it contains contributions from two overlapping peaks. In addition, its intensity is greater at 15 than at 26 °C, reflecting a faster exchange of the dihydrouridine protons compared to those in more stable interactions. The stability of the dihydrouridine interaction in $tRNA^{Phe}_{U39}$ is comparable to that observed for fully modified $tRNA^{Phe}$ as judged from variations in intensity of the resonances with temperature.

An $^1H-^{15}N$ 2D HMQC experiment was employed to overcome the line-width limitations of the difference-decoupling method mentioned above. A refocused pulse sequence is standard for HMQC experiments with a ^1H π pulse midway through the multiple-quantum evolution period (Bax et al., 1983). This experiment has advantages for some systems in that pure absorption line shapes can be obtained, resulting in improved resolution and sensitivity. In our study, application of the refocused HMQC experiment to labeled $tRNA^{Phe}$ resulted in an unacceptable loss in sensitivity because of the short ^1H T_2's for the molecule. Griffey and Redfield (1987) pointed out that the unfavorable CSA component to relaxation destructively interferes for pure heteronuclear zero-quantum coherences, but not in the refocused experiment. We found that a pure zero-quantum HMQC spectrum obtained without a ^1H π pulse gave enhanced resolution and greater sensitivity than possible with the refocused standard HMQC pulse sequence.

$^1H-^{15}N$ 2D HMQC spectra of labeled $tRNA^{Phe}_{U39}$ were acquired at 15 and 26 °C. Spectral resolution is limited in F_2 by the natural line widths and in F_1 by the digital resolution. ^1H chemical shifts are read directly from the F_2 axis. The F_1 axis represents $^1H-^{15}N$ multiple-quantum frequency, and ^{15}N chemical shifts are determined by measuring the horizontal distance from the ^{15}N chemical shift diagonal to the center of the peak.

The spectrum shown in Figure 6 was acquired at 26 °C in order to obtain the best resolution in the crowded "A-U" region. Four resolved peaks are upfield of the principal ^{15}N carrier at 163.7 ppm, while the downfield s^{4}U8 imino resonance with a ^{15}N chemical shift of 179.2 ppm appears as a folded peak. The ^{15}N shift confirmed our assignment of this resonance to s^{4}U8–A14 (Griffey et al., 1983b). Two resolved peaks are seen in the 2D map with nearly identical ^1H chemical shifts of 13.7 ppm. The cross peak with a ^{15}N chemical shift of 156.8 ppm is assigned to the T54-A58 reversed Hoogsteen tertiary base pair. This value is consistent with those from model studies and for T54 in other tRNAs (Griffey, 1984). It is unlikely that other A-U base pairs contribute to the peak at 156.8 ppm because of the unique position normally seen for the ^{15}N chemical shift of T. The proton chemical shift for the N-H unit agrees with the value established by NOE experiments with the fully modified tRNA (Hyde & Reid, 1985). Although the peak for T54 contains a single N-H imino unit, it is more intense than its neighbor at 159.1 ppm, which contains multiple N-H units. In previous studies resonances observed for T54 are typically 1.5–2.0 times more intense than those from A–U interactions (Griffey, 1984). The signal with a ^{15}N chemical shift of 159.1 ppm contains at least two $^1H-^{15}N$ imino units. There are three A–U secondary interactions that could possibly contribute to this cross peak, U12–A23, A7–U66, and U50–A64, although the A7-U66 base pair at the end of the acceptor helix may be absent in the multiple-quantum experiment at 26 °C because of exchange with

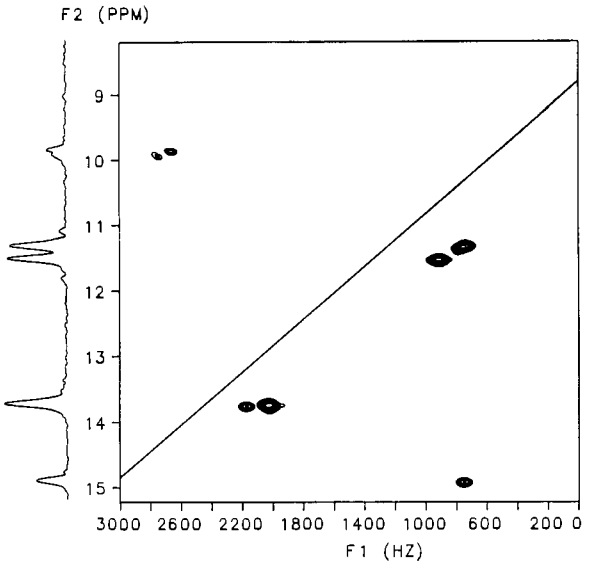

FIGURE 7: $^1H-^{15}N$ 2D HMQC spectrum of [3-^{15}N]uracil-labeled $tRNA^{Phe}_{U39}$ at 15 °C. This experiment results in a 2D plot of ^1H vs $^1H-^{15}N$ multiple-quantum frequency. The spectrum was acquired as described in the legend to Figure 6.

solvent (Griffey et al., 1983b). The other two A-U base pairs are in stable stem regions of fully modified $tRNA^{Phe}$ and should contribute to the 159.1 ppm peak.

The signal with a ^1H chemical shift of 11.55 ppm and a ^{15}N chemical shift of 157.1 ppm was assigned to the Ψ55(N3-H) imino unit on the basis of difference decoupling experiments with fully modified $tRNA^{Phe}$ and is supported by its downfield ^{15}N chemical shift. Model studies with pseudouridine indicate the N(3) nitrogens resonate downfield of unmodified uridines in similar environments (Griffey et al., 1985). The peak with a ^1H chemical shift at 11.33 ppm and an ^{15}N chemical shift at 155.8 ppm is assigned to U51-G63 in analogy with the fully modified tRNA (Hyde & Reid, 1985). Unfortunately, the resonances centered at 9.88 ppm in the difference-decoupling experiment for the dihydrouridine imino protons exchanged too rapidly at 26 °C to be detected by the $^1H-^{15}N$ HMQC experiment.

Several interactions, either predicted from the secondary structure of $tRNA^{Phe}_{U39}$ or detected in the difference decoupling spectrum, were absent in the $^1H-^{15}N$ 2D HMQC spectrum at 26 °C. When the sample was cooled to 15 °C to slow exchange of the imino hydrogens, two new peaks were seen for the dihydrouridines in $tRNA^{Phe}$ (see Figure 7). In the F_2 projection the peaks are barely resolved in the ^1H dimension. This slight difference in chemical shifts is probably responsible for the broadening of the ^1H peak at 9.88 ppm seen in a standard ^1H spectrum. The separation of the peaks in the 2D map is facilitated by differences in their ^{15}N chemical shifts. The ^1H chemical shifts indicate that the dihydrouridines in $tRNA^{Phe}_{U39}$ are paired to the neutral oxygen of a sugar hydroxyl or a bridging water molecule (Davis et al., 1986). In fully modified $tRNA^{Phe}$, the two dihydrouridine interactions were partially resolved. The A31–Ψ39 interaction was assigned to a ^1H peak at 13.20 ppm (Hyde & Reid, 1985). In the $^1H-^{15}N$ spectrum of $tRNA^{Phe}_{U39}$ at 15 °C shown in Figure 8 and in the difference decoupling spectrum, no resonance was observed in the vicinity of 13.2 ppm for an analogous A31–U39 interaction. Thus, the thermally labile Ψ39(N3-H)–A31 interaction was further weakened upon replacing pseudouridine with uridine.

Assignments from NOE Experiments. The ^1H NMR spectrum of fully modified $tRNA^{Phe}$ from *E. coli* strain MA1006/pRK3 was identical with that published by Hyde and Reid (1985). In comparison, the ^1H spectrum of $tRNA^{Phe}_{U39}$ from DD1003/pRK3 (Figure 8) showed minor changes in the region near 13.2 ppm, where the N3 imino hydrogen in the A31-Ψ39 base pair resonates, and at 10.45 ppm, where the peak for the N1 imino proton of Ψ39 (Griffey et al., 1985) was absent. In order to confirm that undermodifications at position 39 did not generate substantial changes in the conformation of $tRNA^{Phe}$, we obtained NOE spectra for comparison with those of the fully modified molecule. The spectra for $tRNA^{Phe}$ and $tRNA^{Phe}_{U39}$ were run in the same buffer used by Hyde and Reid (1985). Five firmly assigned imino resonances [s^4U8, U51, T54, Ψ55(N3-H), and G63] were used as entry points into different regions of the tRNA (Table I).

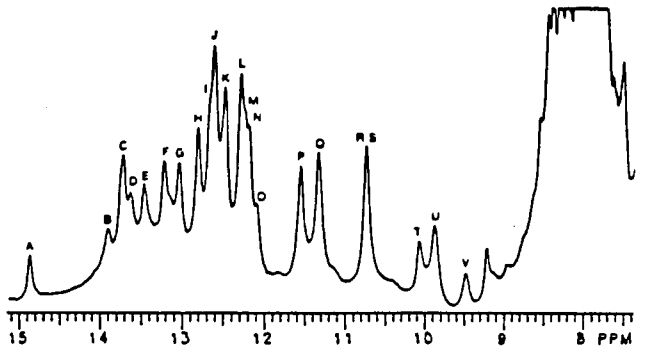

FIGURE 8: ^1H NMR spectrum of $tRNA^{Phe}_{U39}$ at 15 °C.

Table I: Assignments for Pseudouridine-Deficient $tRNA^{Phe}_{U39}$ with ^1H and ^{15}N Chemical Shifts and NOE Connectivities

peak	$^1H/^{15}N$ shift	NOEs	assignment
A	14.87/178.8	G, Ar	s^4U8-A14
B	13.90	J, G, D	CG-11
C	13.73/156.8, 159.1	H, P, Q, T, E, Ar	T54-A58, UA-50
D	13.62	G, B, Ar	UA-12
E	13.46	H, Q, C	CG-49
F	13.22	I, J	CG-2
G, G′	13.03	A, B, D, E, H	m^7G-46, CG-13
H	12.79	A, C, E. L/M	GC-53, GC-5
I	12.75	B, F, L, M, N	GC-3
J	12.62	B, F, L, M, N	GC-10, GC-1, GC-27
K	12.48	L	GC-29
L	12.28	H, J	GC-28, GC-52
M	12.20	H, J	GC-4, GC-6
N	12.17	A, F, G, J, K	GC-30
O	12.08	A, F, G, J, K	G15-C48
P	11.55/157.1	C, L, R/S, T, V	Ψ55N3
Q	11.33/155.8	C, R/S, T, P	UG-51
R/S	10.73	C, L, P	Ψ55N1, Ψ32N1
S	10.68		Ψ32N1
T	10.07	C, Q	GU-63
U	9.88/148.3, 149.8		D16, D20
V	9.50		G18-Ψ55

Dihydrouridine Stem. The s^4U8–A14 interaction assigned to peak A is located at a strategic position in the hinge region of the tRNA molecule with the s^4U8 imino proton positioned near base pairs in the dihydrouridine stem. Irradiation of peak A gives an NOE to peak G and to two aromatic protons at 8.55 and 8.49 ppm (NOE difference spectra are included in the supplementary material). The reversed Hoogsteen geometry for s^4U8-A14 is confirmed by the low-field aromatic NOE at 8.55 ppm assigned to C8-H of A14 (Sanchez et al., 1980). In Watson–Crick A-U base pairs, the uridine imino protons are located close to C2-H protons of adenine which have chemical shifts near 7.5 ppm. The low-field aromatic NOE observed at 8.49 ppm has been seen in spectra for *E. coli* and yeast $tRNA^{Phe}$ and represents an unassigned purine C8-H

proton near s^4U8 and G15. Peak G is assigned to the C13-G22 base pair adjacent to A14. Peak G gives an NOE back to A, and also to B, D, E, and H. Peak G is partially resolved into two resonances, G and B, upon warming the sample to 30 °C. Peak G also contains a contribution from m^7G46, which forms the highly conserved m^7G46–C13–G22 triple interaction (Reid, 1981). Peak D is assigned to U12-A23 on the basis of a strong NOE from G. Irradiation of D gives an NOE back to G, an NOE to B, and an NOE to an aromatic signal at 7.55 ppm. The aromatic NOE confirms the assignment of D to an A-U base pair. Irradiation of B generates NOEs to D, G, and J as shown in the supplementary material, but no aromatic NOE. The small aromatic peak observed in the difference spectrum was a result of spillover to C. Peak B is assigned to C11-G24. The NOE connectivity between B and D was established by experiments at 35 °C where these two resonances are sufficiently resolved to allow observations of mutual NOEs. The NOE from B to J was used to assign peak J to G10-C25. Irradiation of I/J[2] gives an NOE back to B.

TΨC Stem and Loop. The T54–A58 tertiary interaction occupies a strategic position in the tertiary structure of the TΨC loop. It serves as an entry to the TΨC system, analogous to s^4U8 for the D loop and stem, and is adjacent to the highly conserved Ψ55 nucleoside, which forms several critical hydrogen bonds in the TΨC loop. The T54 imino proton was assigned to peak C from ^1H–^{15}N NMR experiments. Peak P was previously assigned to Ψ55(N3-H) on the basis of ^1H–^{15}N NMR experiments. Irradiation of C as shown in the supplementary material affects E, H, P, Q, T, and aromatic peaks at 8.31 and 7.53 ppm. The chemical shift of the aromatic NOE at 8.31 ppm is typical of a C8-H purine proton and is assigned to C8-H of A58. The aromatic NOE at 7.53 ppm is typical of an adenine C2-H proton and confirms the contribution of an additional A-U base pair to peak C. The NOE to P, assigned to Ψ55(N3-H), further corroborates assignments for T54 and Ψ55. Irradiation of P gives an NOE back to C and also to L, R/S, T, and V. Peak V is assigned to G18 on the basis of the crystal structure of yeast $tRNA^{Phe}$, which suggests a hydrogen bond between G18 and Ψ55, and in analogy with the assignment in fully modified $tRNA^{Phe}$ (Hyde & Reid, 1985). The NOE from P to peak R/S shows R/S contains Ψ55(N1-H). The chemical shift of R/S is nearly identical with that of Ψ55(N1-H) in fully modified $tRNA^{Phe}$ assigned by Griffey et al. (1985). Peak L contains G52–C62 as indicated by the NOE from P, an interaction established by Hyde and Reid (1985) for the fully modified tRNA. Similarly, the NOE from C to H indicates that peak H contains G53-C61. This assignment is further strengthened by NOEs from H to L/M and an NOE from L/M back to H. The large mutual NOEs between peaks Q and T permit their unambiguous assignments as the imino protons in the U51-G63 base pair. From ^1H–^{15}N NMR data, we assign Q to U51 and T to G63. Peak Q, but not T, gives a NOE to Ψ55(N3-H) while Ψ55(N3-H) gives a NOE to T and not Q. Irradiation of Q affects C, as does irradiation of T. In turn, irradiation of C affects both Q and T. Peak C thus contains the neighboring U50-A64 base pair. An NOE from C to E continues the trail of NOE connectivities and permits us to assign E to the C49-G65 base pair at the internal end of the TΨC stem.

Acceptor and Anticodon Stems. Assignment of the acceptor and anticodon stems was a particularly formidable task due to the large number of consecutive G-C base pairs in both regions. In fully modified $tRNA^{Phe}$, a convenient entry into the anticodon stem was provided by the easily assigned A31-Ψ39 base pair. Substitution of uridine for pseudouridine in $tRNA^{Phe}_{U39}$ destabilized the A31–U39 interaction, and the U39 imino proton was not detected in any of our ^1H–^{15}N NMR experiments. The acceptor and anticodon stems were assigned from the NOE patterns between G-C base pairs, and by analogy with assignments from fully modified $tRNA^{Phe}$ (Hyde & Reid, 1985).

Irradiation of peak F affects I and J. Peak F is assigned to C2-G71, peak I to C3-G70, and peak J to G1-C72. Peaks I and J have nearly coincident chemical shifts, and when irradiated affect B, F, L, M, and N. Peak M contains G5-C68 as well as the previously assigned G53-C61. Irradiation of H gives a strong NOE back to L/M, leading to the assignment of L/M as containing G6-C67, as well as C4-G69. NOE connectivities involving A7-U66 were not detected, presumably due to the lability of this interaction at the end of the helix.

Assignments in the anticodon stem were based on a similar strategy. Peak O was thought to contain G15-C48, in analogy with fully modified $tRNA^{Phe}$. Irradiation of N/O gives an NOE to peak A, confirming the assignment. As shown in the supplementary material irradiation of N/O also gives NOEs to F, G, J, and K. Peak N contains G30-C40 and gives an NOE to peak K, which contains G29-C41. Irradiation of K affects L, and irradiation of L/M affects H, J, and K. Peak L is assigned to G28-C42, and J is assigned to G27-C43. All of the imino protons from G-C base pairs in the anticodon stem were assigned, but peaks J, K, L, M, and N contain more than one resonance. While the assignments are consistent with the NOE patterns, no independent corroboration of the results was available.

Variable-Temperature Studies of Pseudouridine ^1H Imino Resonances in $tRNA^{Phe}$ and $tRNA^{Phe}_{U39}$. The effect of temperature on ^1H imino resonances of Ψ32, Ψ39(U39), and Ψ55 was studied with identical samples of $tRNA^{Phe}$ and $tRNA^{Phe}_{U39}$. For both molecules, the N1-H imino hydrogen bonds were surprisingly strong. In $tRNA^{Phe}$ the signal for the Ψ32(N1-H) melted at 42 °C, while the two remaining N1 imino resonances for Ψ39 and Ψ55, along with peaks for secondary A-U hydrogens, did not disappear until the temperature was raised above 55 °C. In contrast, the N3-H signal for the A31-Ψ39 pair had melted by 25 °C. ^1H NMR spectra of $tRNA^{Phe}_{U39}$ were virtually identical with those for fully modified tRNA except for the imino hydrogens of U39. As expected, the peak at 10.45 ppm, assigned to Ψ39(N1-H) in $tRNA^{Phe}$ (Griffey et al., 1985), was missing. However, we also no longer saw a peak for U39 corresponding to the N3 imino resonance in the A31-Ψ39 base pair at the end of the anticodon stem. A signal assignable to an A31–U39 interaction was not detected in ^1H, ^1H–^{15}N HMQC, or ^1H–^{15}N difference decoupled spectra of $tRNA^{Phe}_{U39}$ at temperatures down to 5 °C.

Discussion

Pseudouridine synthase I, encoded by *hisT*, catalyzes the intramolecular rearrangement of uridine to pseudouridine at positions 38–40 in *E. coli* tRNAs (Turnbough et al., 1979; Kammen et al., 1988). Although mutants lacking pseudouridine synthase I activity are viable, they exhibit several biochemical deficiencies including reduced growth rates, increased error levels during translation, and reduced polypeptide chain elongation rates (Parker, 1982; Palmer et al., 1983). This latter phenomenon is also responsible for derepression of several biosynthetic operons regulated by transcription-attenuation mechanisms (Landick & Yanofsky, 1987). Presumably two other enzymes are responsible for Ψ modifications

[2] Denotes overlapping peaks.

at positions 32 and 55. Recently Samuelsson and Olsson (1990) found three distinct pseudouridine synthase activities in yeast for modification of tRNA at positions 13, 39, and 55.

In *E. coli* $tRNA^{Phe}$, undermodification of *hisT* mutants occurs exclusively at position 39 and leaves an A31-U39 base pair at the end of the anticodon stem (Kammen et al., 1988). A comparison of ^1H NMR spectra for $tRNA^{Phe}_{U39}$ and $tRNA^{Phe}$ indicates that the two molecules have very similar structures in solution. With the exception of features specifically related to Ψ39, the ^1H chemical shifts and NOE connectivity patterns of the tRNAs are virtually identical. Both imino protons in Ψ39 participate in hydrogen bonds in $tRNA^{Phe}$. The N3 hydrogen interacts with A31 to form a Watson–Crick base pair, while N1-H interacts with a neutral sp^3 hydrid oxygen typical of those in sugar hydroxyls or tightly bound waters. Similar interactions are seen for the N1 protons in Ψ32 and Ψ55.

The N1 hydrogens for Ψ32, Ψ39, and Ψ55 in $tRNA^{Phe}$ are surprisingly stable relative to other imino hydrogens in the molecule. They exchange slowly with water, and their signals persist at temperatures where imino protons of secondary Watson–Crick A-U pairs in the centers of stems melt. In the pH 7 buffer we used, the rate constant for exchange of imino hydrogens exposed to bulk water, k_{ex}, can be estimated from eq 1 where α is the accessibility factor, k_d is the diffusion-

$$k_{ex} = \alpha k_d[B]\left(\frac{10^{\Delta pK_a}}{1 + 10^{\Delta pK_a}}\right) \quad (1)$$

limited collision rate constant, [B] is the concentration of base catalyst, and ΔpK_a is $pK_a^{acceptor} - pK_a^{donor}$ (Eigen, 1964). The estimated k_{ex} value of $\sim 10^3$ s^{-1} for hydroxide-catalyzed exchange of imino hydrogens at pH 7, where $\alpha \sim 1$, $pK_a^{donor} \sim 9$, and $k_d \sim 10^{10}$ s^{-1}, would result in averaging of the imino resonances with those of the solvent. Access of water to those imino hydrogens that give discrete resonances is presumably impeded. Typically, slow exchange is an indication that the protons are inaccessible because they are immobilized in hydrogen bonds, and a rate-limiting conformational change is required before exchange with solvent (Hilbers, 1979; Marshall & Wu, 1990).

While there are no X-ray structures of *E. coli* $tRNA^{Phe}$, the yeast molecule, for which structures do exist, also has an A31–Ψ39 interaction at the bottom of its anticodon stem and, of course, a conserved TΨC loop. It also gives discrete stable ^1H resonances for its N1 imino hydrogens (Leroy et al., 1985; Hyde & Reid, 1985). Inspection of molecular graphics models[3] of yeast $tRNA^{Phe}$ created from atomic coordinates based on X-ray structures (Ladner et al., 1975; Quigley et al., 1975; Sussman & Kim, 1976; Westhof & Sundaralingham, 1986) indicates that the N1 imino hydrogens of Ψ39 and Ψ55 cannot form hydrogen bonds directly to other heteroatoms in the tRNA. Furthermore, the hydrogens are not shielded from the solvent. However, the distances between the pseudouridine N1 imino hydrogens and oxygens in the pseudouridine 5′-phosphates are ideal for intramolecular bidentate chelation of a water molecule by the nucleotide. A stable cyclic water-bridged structure is consistent with our NMR data. In addition, slowly exchanging protons in pseudouridines are a common feature in other tRNAs. Thus far, N1 imino resonances have been identified in ^1H NMR spectra of *E. coli* $tRNA^{Phe}$ (Griffey et al., 1985a), *E. coli* $tRNA_f^{Met}$ (Griffey et al., 1985a), *E. coli* $tRNA^{Glu}$ (Griffey et al., 1985a), *E. coli* $tRNA^{Tyr}$ (Griffey et al., 1985a), *E. coli* $tRNA^{Val}$ (Choi, 1985), *E. coli* $tRNA^{Gln1}$ (Redfield et al., 1986), yeast $tRNA^{Phe}$ (Roy et al., 1984) and *Thermoactinomyces thermophilus* $tRNA^{Ile}$ (Choi & Redfield, 1986). With the exception of Ψ38 and Ψ39 in *E. coli* $tRNA^{Gln1}$, distinct NMR peaks are seen for N1 imino hydrogens in pseudouridines at positions 12, 32, 39, and 55.

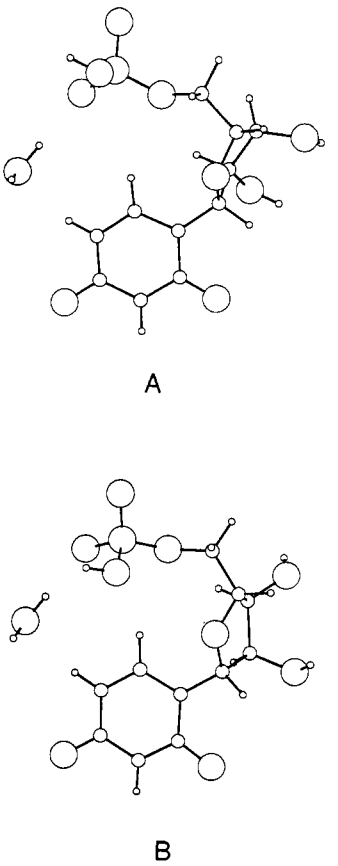

FIGURE 9: Structures for pseudouridine with a hydrogen–water bridge between the N1 imino hydrogen and an oxygen in the 5′-phosphate. (A) 2′-Endo ribosyl conformer; (B) 3′-endo ribosyl conformer.

The pseudouridine modification creates a nucleotide in which the basic topology, including all of the hydrogen-bonding features, of uridine is preserved, except for the new imino moiety at the original C5 position. This additional locus for hydrogen bonding points toward the 5′-phosphate and accommodates a water bridge, as proposed above, when the nucleoside is in an anti conformation. Molecular modeling indicates that several different low-energy cyclic bridged structures are possible by making slight adjustments in the C4′–C5′ and C5′–O, and O–P torsional angles. As illustrated in Figure 9, this includes 2′-endo (A) or 3′-endo (B) ribosyl conformers.

A related intranucleotide water bridge was recently proposed by Topiol et al. (1990) as a means for selectively stabilizing the syn conformer of cyclic GMP. They noted that the X-ray structure of the sodium salt of cGMP has a bridging water molecule in the sodium/water cluster that forms a bridge between a 2-amino hydrogen and the axial phosphate oxygen. Their calculations suggested that the hydrogen-bonding network increases the preference of the syn versus the anti conformation of cGMP by approximately 2 kcal/mol. Similar degrees of stabilization would be expected for water bridges in pseudouridine.

Exchange rates of most imino protons in tRNAs with water are subject to buffer catalysis, and the lifetime, τ_{ex} of the

[3] Modeling was conducted on an IRIS GT80 workstation with Biograf software. Atomic coordinates were from the Brookhaven library.

hydrogen-bonded complex, where $\tau_{ex} = k_{ex}^{-1}$, is determined by extrapolation of k_{ex} to infinite buffer concentration. In their study of yeast $tRNA^{Phe}$, Leroy et al. (1985) discovered the lifetime of the N3–phosphate interaction for Ψ55 was 100 ms at 27 °C in the presence of magnesium. In contrast, τ_{ex} was $\ll$1 ms for the N1 hydrogen of the same base. Yet, in our study, the hydrogens at N1 and N3 melt at similar temperatures in the absence of base catalysis. We suggest that this difference is characteristic of directly hydrogen-bonded and water-bridged structures. In the former case, base catalysis of exchange cannot occur until the hydrogen bond between the N3 hydrogen and the phosphate oxygen is ruptured. Here, the extrapolated value for τ_{ex} is a measure of the intrinsic lifetime of the base pair. However, the base can *catalyze* rupture of the stable N1–H_2O–phosphate interaction by removing the nonbonded proton on the bridging water. The resulting hydroxide bridge should rapidly deprotonate N1, thereby positioning a water molecule between two negatively charged moieties and facilitating exchange of the hydrogen originally at N1 with bulk solvent.

The only observable effect of undermodification at position 39 in $tRNA^{Phe}_{U39}$ is to weaken the interaction with A31 at the base of the anticodon stem, with a concomitant lowering of the melting temperature for the N3 imino signal by at least 20 °C. The absence of a signal for U39(N3-H) may be due to fraying by a rapid equilibrium between the native conformation and a minor conformer with a disrupted A31-U39 pair or due to a major conformational change resulting in an expanded anticodon loop. Since the anticodon loop extends downward from the rest of the tRNA, a change of this type would not be expected to substantially alter the remaining imino resonances in the molecule. A significant increase in the equilibrium population of conformers with a nine-membered anticodon loop might well affect aminoacylation and polypeptide chain elongation rates for reactions involving $tRNA^{Phe}$. In the absence of unexpected differences in stacking interactions for U and Ψ, the enhanced stability of the A31-Ψ39 base pair must reside in additional stabilization through N1.

Ψ55, the most highly conserved of all tRNA modifications, is located at a sharp bend in the phosphodiester backbone in the hinge region. Other U → Ψ modifications are commonly encountered at the junctions between stems and loops where the local structure is normally under stress. We suggest that a major role of the U → Ψ modification is to further stabilize the anti conformer by creating a site for formation of an intranucleotide water bridge, thereby reducing the flexibility of the tRNA in the vicinity of the modification.

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Supplementary Material Available

Three figures showing NOE difference spectra for $tRNA^{Phe}_{U39}$ at 21 °C with Figure S1 indicating irradiation of peaks A, B, C, and T; Figure S2 indicating irradiation of peaks D, E, F, G, H, and I/J; Figure S3 indicating irradiation of peaks K, L/M, O, P, Q, and R/S (4 pages). Ordering information is given on any current masthead page.

Registry No. Ψ, 1445-07-4; adenine, 73-24-5; water, 7732-18-5.

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