Assignment of the Low-Field ^{1}H NMR Spectrum of *Escherichia coli* tRNA^{Phe} Using Nuclear Overhauser Effects†

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ABSTRACT: The imino region of the proton NMR spectrum of *Escherichia coli* tRNA^{Phe} has been largely assigned from the nuclear Overhauser effects between neighboring bases. These have led to the unambiguous assignment of the imino protons of the ribothymidine stem and of most of the dihydrouridine stem of this tRNA and given several other sets of connectivities. These connectivities are discussed in reference to the previously reported temperature studies of the spectrum [Hurd, R. E., & Reid, B. R. (1980) *J. Mol. Biol. 142*, 1981] and compared with assignments of other tRNAs resulting in tentative assignments of the rest of the spectrum.

The extreme low-field region of the proton NMR[1] spectra of tRNAs contains resonances from the hydrogen-bonded imino protons and provides information on the helical stems and tertiary base pairing. From studies of these spectra, detailed structural and dynamic data of the molecule under different solution conditions can be obtained (Reid, 1981). The first step in this process is the assignment of the observed peaks. Previous methods of assigning these resonances include chemical modification of the tRNA (Reid et al. 1975, 1977; Daniel & Cohn, 1975; Wong et al., 1975; Hurd & Reid, 1979a,b), use of helical fragments (Lightfoot et al., 1973; Kearns & Shulman, 1974; Reid et al., 1979), and ring-current calculations (Shulman et al., 1973; Robillard et al., 1976; Geerdes & Hilbers, 1977; Reid et al., 1979). More recently, the nuclear Overhauser effect (NOE) has proved useful in the unambiguous assignment of resonances (Johnston & Redfield, 1978, 1981; Hurd & Reid, 1979a; Tropp & Redfield, 1981; Hare & Reid, 1982a,b). The NOE occurs between two nuclei that are close in space; if the first nucleus is saturated by irradiation, there is an effect on the intensity of the resonance of the neighboring nucleus (Noggle & Shirmer, 1971). For protons in large molecules, the effect is a decrease in intensity, a negative nuclear Overhauser effect (Bothner-By & Noggle, 1979). The small NOEs between hydrogen bonds of adjacent base pairs, together with NOEs to proximal protons in the same base pair, lead to the sequential assignment of the spectra of tRNAs from a known starting point (Roy & Redfield, 1981; Roy et al., 1982a,b; Hare & Reid, 1982a,b; Heerschap et al., 1982). In order to examine the effect of sequence on the structure of tRNAs, it is necessary to assign the proton NMR spectra of several different tRNAs. This paper presents the results of an NOE study on *Escherichia coli* tRNA^{Phe}.

Materials and Methods

E. coli tRNA^{Phe} was purified to homogeneity from unfractionated RNA by chromatography of crude *E. coli* tRNA on benzoylated DEAE-cellulose at pH 5.0 (Gillam et al., 1967) followed by chromatography on DEAE-Sephadex at pH 7.5 (Nishimura, 1971). The purified tRNA accepted 1800 pmol of phenylalanine/A_{260} unit when aminoacylated with partially purified phenylalanyl-tRNA synthetase. For the NMR studies, 7–10 mg of tRNA was dissolved in 0.3 mL of buffer containing 10 mM cacodylate, 100 mM NaCl, and 1 mM EDTA at pH 7.0. A 50-μL aliquot of D_2O was added as a lock signal and 0.2 mM DSS as an internal reference. The magnesium concentrations in these samples are detailed below.

Proton NMR spectra were collected on a Bruker WM500 FT NMR spectrometer with 16-bit digitizer resolution, using a modified 21412 Redfield pulse sequence (Redfield, 1978). The spectra were collected in 8K channels with a sweep width of 14000 Hz, a pulse length of 205 μs, and a carrier offset 4730 Hz downfield of water. Nuclear Overhauser effects were measured by collecting sets of 16 scans with the decoupler alternately on- and off-resonance. The two FIDs were stored separately and subsequently subtracted. In this way, several difference spectra could be obtained under the same conditions with one reference spectrum. Typically, 4000 scans were collected for each experiment. A saturation pulse of between 0.6 and 1.2 s was used, and the decoupler power was adjusted to just saturate the target resonance with minimal effects on the neighboring peaks. Spectra were integrated by using a Numonic Graphic Corp. calculator.

Results

The sequence of *E. coli* tRNA^{Phe} (Barrell & Sanger, 1969) is shown in Figure 1 arranged in the classical cloverleaf form showing the secondary base pairing. In the X-ray crystal structure of yeast tRNA^{Phe}, several tertiary base pairs were also found (Kim et al., 1974; Robertus et al., 1974). Most of these tertiary interactions involve bases that are invariant or semiinvariant in different tRNAs and so are thought to be present in all tRNAs (Klug et al., 1974; Kim et al., 1974). These tertiary base pairs are shown as solid connecting lines.

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[1] Abbreviations: D, dihydrouridine; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; EDTA, ethylenediaminetetraacetic acid; FID, free-induction decay; m^{7}G, 7-methylguanosine; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; ppm, parts per million; s^{4}U, 4-thiouridine; T, ribothymine; tRNA, transfer ribonucleic acid; Ψ, pseudouridine.

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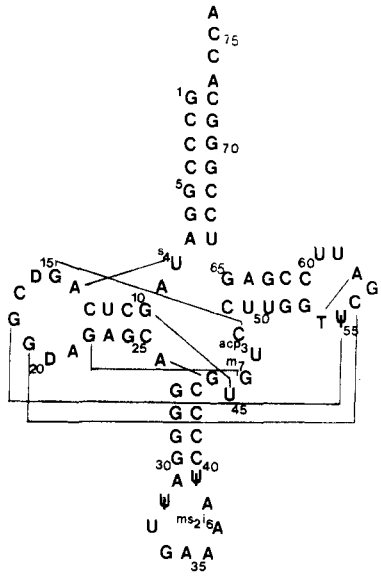

FIGURE 1: Nucleotide sequence of *E. coli* $tRNA^{Phe}$ arranged in the classical cloverleaf structure (Barrell & Sanger, 1969) showing the tertiary hydrogen bonds seen in the yeast $tRNA^{Phe}$ crystal structure (Kim et al., 1974).

E. coli $tRNA^{Phe}$ contains several consecutive GC or CG base pairs, and thus, the imino region of its spectrum has many close or overlapping resonances, even at 500 MHz. The spectrum obtained at 25 °C in buffer containing 12 mM magnesium is shown in Figure 2a. The peaks are labeled alphabetically from left to right. Resonances that resolve from multiple peaks under different solvent conditions are given the prime notation, e.g., peaks B′ and L′. Integration of the peak areas at various magnesium concentrations and low temperature indicates that peak B contains at least four proton resonances as does the D–E–F complex while the K–L–M complex contains five resonances and peak N three. Optimum resolution of the various multiple peaks occurs at different magnesium concentrations and temperatures; thus, NOE assignment experiments were performed under different conditions depending on which peak was being studied. Most assignments were repeated under several conditions.

Ribothymidine Helix. A convenient starting point for the NOE assignment studies of tRNAs is the methyl group of the sole ribothymine residue T-54. Transfer RNAs have relatively few methyl groups, so the thymidine methyl resonance is easily identified. This resonance is close to 1 ppm, far upfield of the imino region (Kan et al., 1974; Kastrup & Schmidt, 1975; Davanloo et al., 1979), and so it can be saturated without directly affecting the latter protons (Tropp & Redfield, 1981). On irradiation of the thymine methyl resonance at minimal magnesium ion concentration, the only peak affected in the imino region is peak U. At 12 mM magnesium, effects are seen on peaks Q, H, N, and B as well as on U (Figure 2b). By referring to the crystal structure of yeast $tRNA^{Phe}$ (Kim et al., 1974), the N1-H proton of Ψ-55 is predicted to be the closest imino proton to the T-54 methyl group. Peak U having the largest NOE from the thymidine methyl group is thus assigned to Ψ-55 N1-H in agreement with the results of Tropp & Redfield on yeast $tRNA^{Phe}$ (Tropp & Redfield, 1981) and Hare & Reid on *E. coli* $tRNA^{Ile}$ (Hare & Reid, 1982b). The resonance position of peak U at 10.6 ppm, upfield of the majority of imino protons, suggests that this proton is not hydrogen bonded. Irradiation of peak U affects peak Q and also an aromatic peak at 7.25 ppm, presumably the Ψ-55 C6-H proton (Figure 3b) (Tropp & Redfield, 1981). The latter effect confirms the assignment of U as Ψ-55 N1-H, and Q

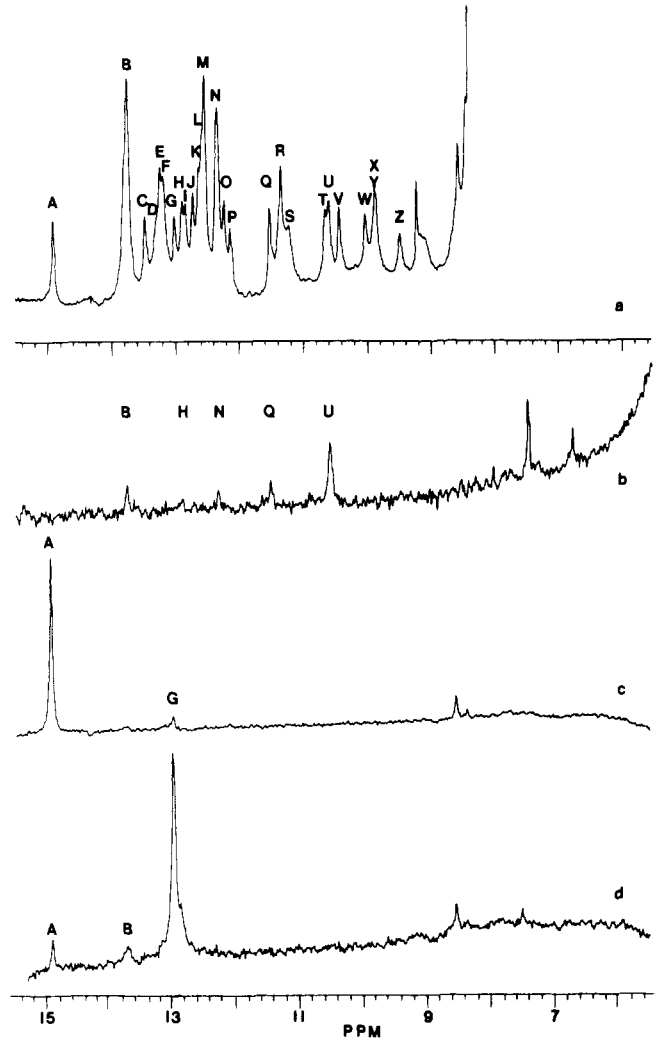

FIGURE 2: (a) Low-field ^1H NMR spectrum of *E. coli* $tRNA^{Phe}$ in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 12 mM magnesium at 25 °C together with the NOE difference spectra obtained on irradiation of (b) the thymidine methyl resonance at 0.98 ppm for 0.4 s, (c) peak A, the ring NH of s^4U-8-A-14, for 0.6 s, and (d) peak G for 0.6 s.

is assigned to the remaining imino proton of Ψ-55, namely, the N3-H proton.

Irradiation of peak Q (Ψ-55 N3-H) affects peaks B and Z and, more weakly, peak U (Figure 3C). Irradiation of peak Z affects peaks Q and B (not shown). From its chemical shift of 9.5 ppm near the amino region, peak Z may be the G-18 imino proton (not hydrogen bonded) or the G-18 amino proton hydrogen bonded to Ψ-55. As both the T-54 methyl resonance and Ψ-55 N3-H give NOEs to peak B, peak B is assigned to the imino proton of the T-54-A-58 tertiary base pair, a Hoogsteen base pair, stacked between G-18-Ψ-55 and GC-53 at the end of the ribothymidine helix in the yeast $tRNA^{Phe}$ crystal structure. Peak B is a multiple peak, containing at least three additional resonances besides T-54-A-58. At lower magnesium ion concentrations, one of the resonances in B, peak B′, separates from the main peak. Under these conditions it is B and not B′ that is affected by irradiation of Q and that therefore contains T-54-A-58. At low magnesium concentrations, irradiation of the complex peak B affects several peaks including Q, confirming that it contains T-54-A-58 (Figure 4c). Peak B also cross-saturates H and, more weakly, N, which are both affected by irradiation of the thymine methyl group and which therefore both contain resonances of protons in the ribothymidine stem. From the NOE from B, either H

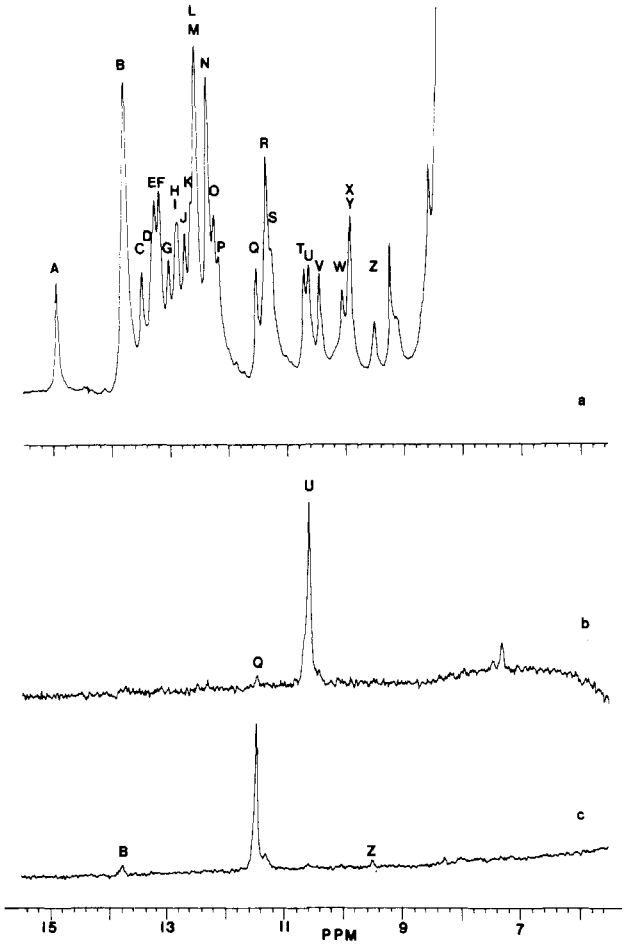

FIGURE 3: (a) Low-field ^{1}H NMR spectrum of *E. coli* tRNA^{Phe} in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 12 mM magnesium at 17 °C together with the NOE difference spectra obtained on irradiation of (b) peak U for 0.4 s, and (c) peak Q for 0.6 s.

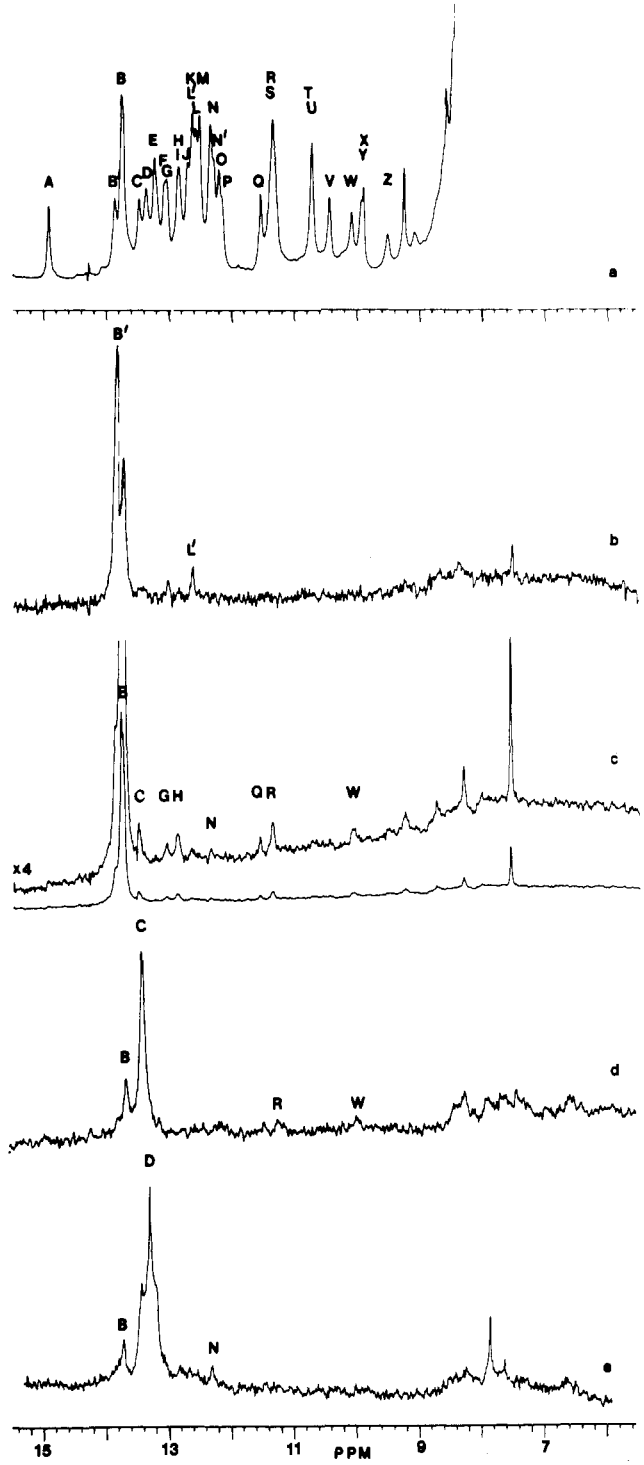

FIGURE 4: (a) Low-field ^{1}H NMR spectrum of *E. coli* tRNA^{Phe} in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 5 mM magnesium at 12 °C together with the NOE difference spectra obtained on irradiation of (b) peak B′ for 0.6 s, (c) peak B for 0.6 s, (d) peak C for 1.5 s (to obtain second order NOEs), and (e) peak D for 0.6 s.

or N could be the imino proton next to T-54-A-58. However, both B and N contain multiple resonances so that the NOE between B and N may come from a different pair of resonances, unrelated to T-54-A-58. H is a single proton resonance in the ribothymidine stem, and irradiation of peak H affects peak B and peak N (Figure 5b). Thus H is assigned to GC-53, and N contains CG-52. On irradiation of the complex peak N effects can be seen on several peaks including H, confirming the assignment of CG-52 in peak N (Figure 5d). Thus, the sequential connectivities, together with the observation that B, H, N, and Q are all close to T-54 (Figure 2b), can only be rationalized by assigning the imino protons Q, B, H, and N to Ψ-55, T-54-A-58, GC-53, and GC-52, respectively.

The next base pair in the sequence is UG-51, the only GU wobble base pair in the molecule. A wobble base pair contains two imino protons within 3 Å of each other, and so these protons are easily assigned by their strong mutual NOEs (Johnston & Redfield, 1981). Irradiation of peak R for only 0.2 s gives a strong NOE to peak W and vice versa, showing that they are resonances from a GU base pair. Irradiation of R or W for longer times gives effects at B and more weakly at N (Figure 6). At lower magnesium concentrations the NOE from R or W is to the complex B peak and not to the resolved B′. Thus, peak B contains the UA-50 imino proton resonance as well as T-54-A-58. As B is a multiple peak, the other neighbor of UA-50 cannot be unambiguously determined from the remaining NOEs of peak B; it could be C, G, or N (Figure 4c). The ambiguity is resolved by the observation of a weak secondary NOE at long irradiation times from peak C to the wobble imino resonances R and W of UG-51 (Figure 4d), as well as a strong first-order NOE to peak B. Thus peak C is assigned to CG-49. The T helix thus generates the nine resonances C, B, R + W, N, H, B, and Q + U from base pairs 49, 50, 51, 52, 53, 54, and 55, respectively.

Dihydrouridine Stem. A second independent starting point for assignment of the low-field proton NMR spectra of *E. coli* tRNAs is the s^{4}U-8-A-14 tertiary hydrogen bond. Because

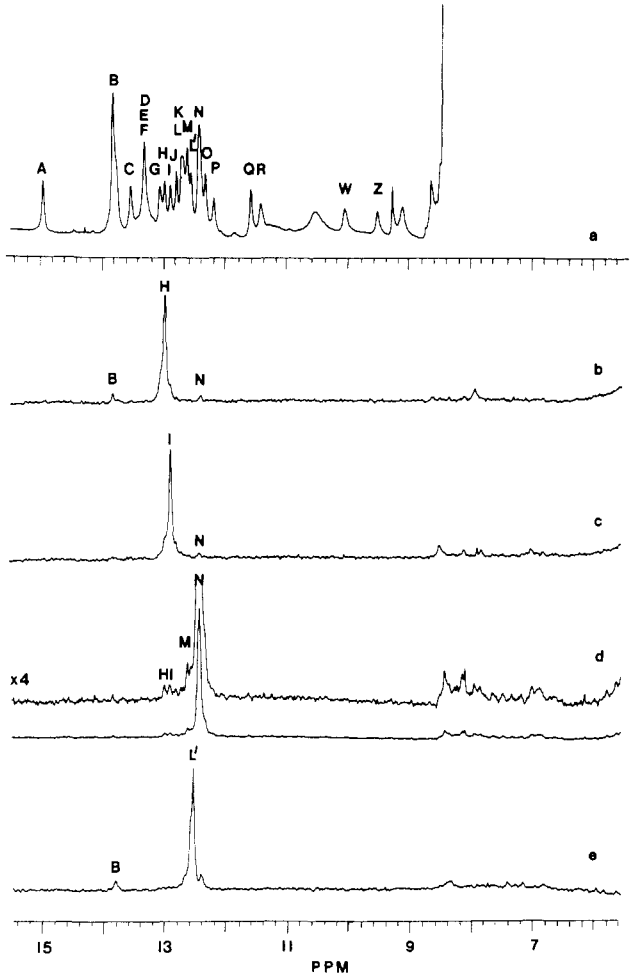

FIGURE 5: (a) Low-field ^{1}H NMR spectrum of *E. coli* tRNA^{Phe} in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 25 mM magnesium at 37 °C together with the NOE difference spectra obtained on irradiation of (b) peak H for 1.0 s, (c) peak I for 1.0 s, (d) peak N for 1.0 s, and (e) peak L′ for 1.0 s.

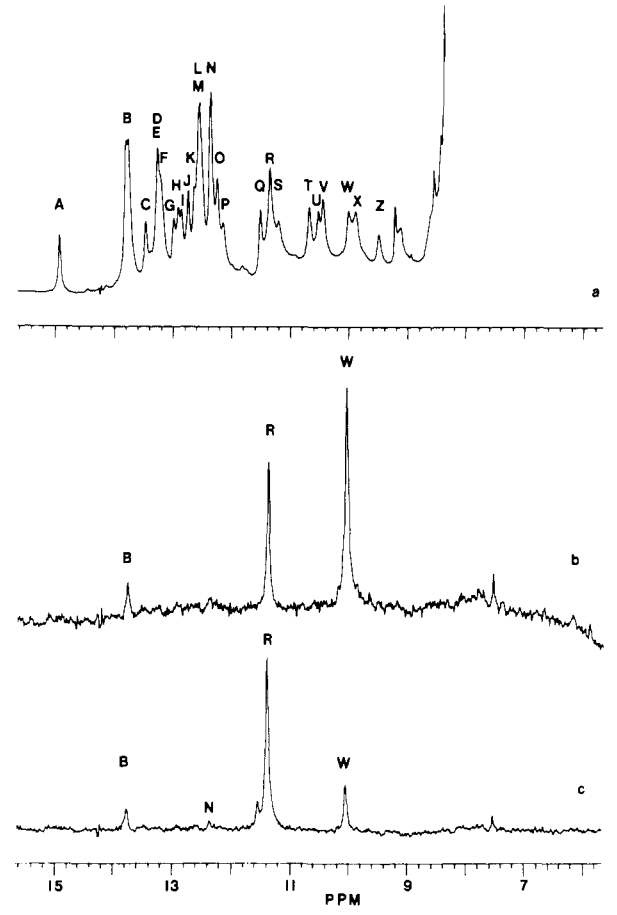

FIGURE 6: (a) Low-field ^{1}H NMR spectrum of *E. coli* tRNA^{Phe} in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 15 mM magnesium at 17 °C together with the NOE difference spectra obtained on irradiation of (b) peak W for 1.0 s and (c) peak R for 1.0 s.

of the deshielding effect of the sulphur atom, this imino proton resonates further downfield than the other AU hydrogen bonds as shown by dethiolation experiments (Hurd & Reid, 1979a; Wong et al., 1975) and by s^{4}U-8 spin-labeling (Daniel & Cohn, 1975). Peak A at 14.9 ppm can be confidently assigned to this resonance, which decreases to 0.5 intensity in tRNA^{Phe} samples containing 50% s^{4}U and 50% U at position 8 (unpublished observations). Irradiation of peak A affects peak G (Figure 2c). Peak G is assigned to CG-13, which is stacked under U-8-A-14 in the X-ray crystal structure of yeast tRNA^{Phe}. After longer irradiation, small effects from peak A are also seen to peaks B and P. Peak P gives a weak NOE back to A but to no other imino peaks (Figure 7d). P is thus tentatively assigned to the tertiary base pair G-15-C-48 located somewhat more distantly on the outer side of s^{4}U-8-A-14. This reverse Watson–Crick tertiary base pair has been assigned in *E. coli* tRNA^{Val} at 12.25 ppm by paramagnetic ion effects (Hurd et al., 1979) and by NOE from s^{4}U-8-A-14 (Hare, 1983). Irradiation of peak G, CG-13, affects both peak A and peak B (Figure 2d). At low magnesium ion concentrations, it is again B, not the resolved B′, that is affected on irradiation of peak G. Thus, peak B contains UA-12 as well as the T-54-A-58 and UA-50 resonances shown in the previous section. Peak B gives NOEs to C, G, H, Q, and R, which all have been assigned, and also more weakly to N (Figure 4c, the small effect to L′ is from the neighboring B′). It is possible that N contains CG-11; however, peak B may contain a fourth unassigned peak; thus, the assignment of CG-11 is ambiguous from the NOE data.

Other NOEs. (*A*) *Resonances in the Far Low Field and Their Neighbors.* There are two remaining unassigned AU-type base pairs in the molecule, namely, AU-7 in the acceptor stem and AΨ-31 in the anticodon stem. Peak D at 13.4 ppm can be confidently assigned to an AU resonance from its low-field shift and its strong NOE to a sharp aromatic resonances at 8.05 ppm (Figure 4e), (Sanchez et al., 1980). No such NOEs to adenine C2-H protons are seen from any other peaks besides B′, B, and D. Thus, the other AU resonance is in peak B′ or B. Peak B′, a single proton, appears to give an NOE to an aromatic peak at 7.5 ppm (Figure 4b); however, this may be from spillover saturation of the neighboring peak B. At low magnesium concentrations, peak B′ is further resolved from peak B and does not give this aromatic NOE although it does still give an NOE to L′ (M. Woods, personal communication). Peak B′ is probably from a GC base pair, and peak B may contain the other AU imino proton resonance. The chemical shift of peak D at high magnesium concentrations, 13.2 ppm, is identical with that of AΨ-31 in yeast tRNA^{Phe} (Roy et al., 1982b), and on this basis, we favor resolving the ambiguity in assignment between AU-7 and AΨ-31 by assigning peak D to AΨ-31. In both yeast tRNA^{Phe} and *E. coli* tRNA^{Phe} AΨ-31 is directly below a GC base pair, but in yeast tRNA^{Phe} the C is methylated. If peak D is in the anticodon stem then, by elimination, peak B contains AU-7.

The NOEs from peak B have been discussed above. Peak B′ gives an NOE to L′ (Figure 4b). That the NOE is to L′

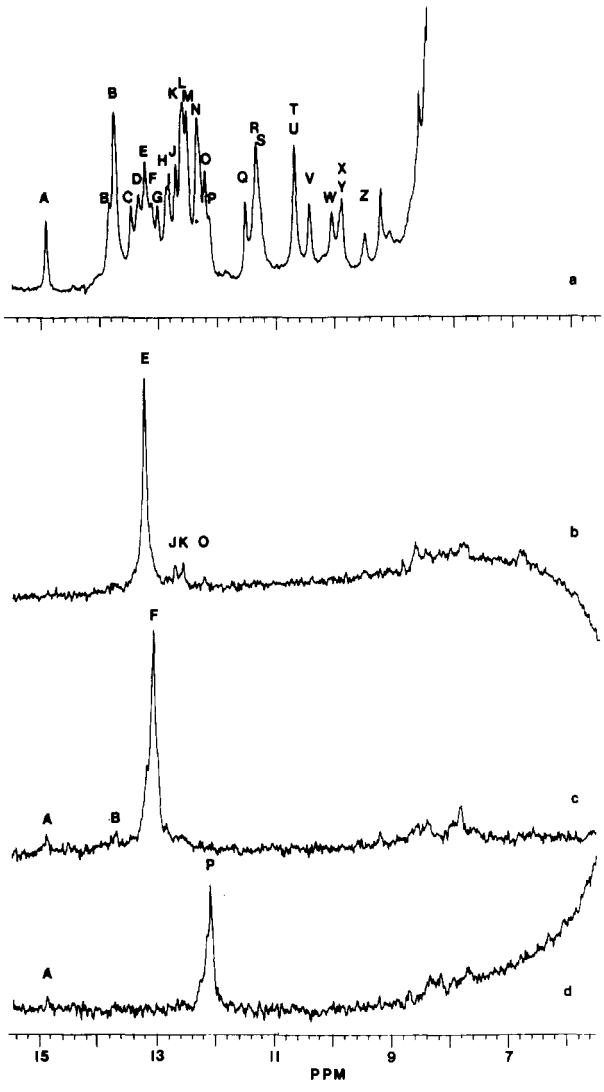

FIGURE 7: (a) Low-field ^{1}H NMR spectrum of *E. coli* tRNA^{Phe} in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 6 mM magnesium at 17 °C together with the NOE difference spectra obtained on irradiation of (b) peak E for 1.0 s, (c) peak F for 0.8 s, and (d) peak P for 1.2 s.

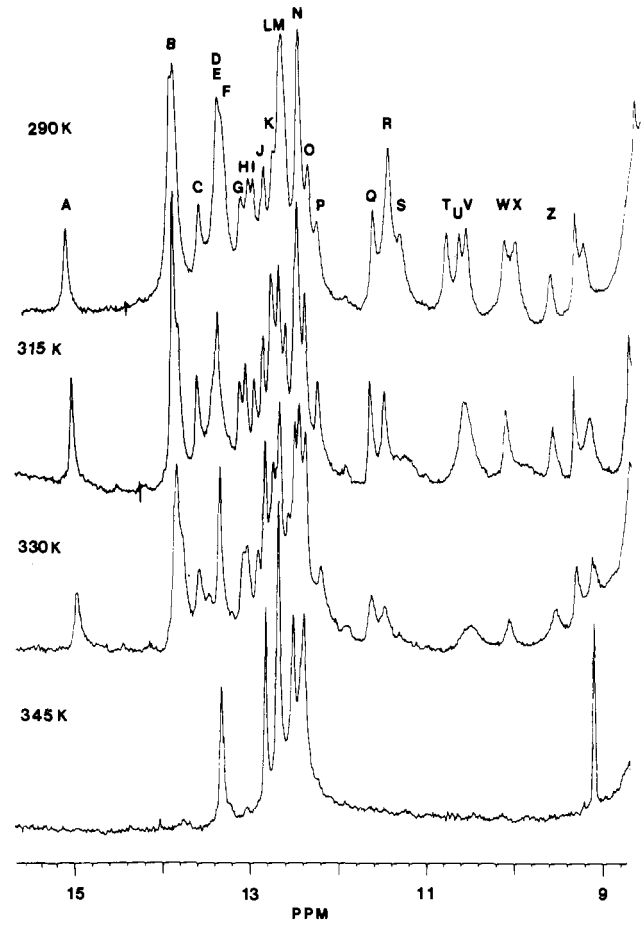

FIGURE 8: Low-field ^{1}H NMR spectrum of *E. coli* tRNA^{Phe} in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 15 mM magnesium. From top to bottom, the spectra were taken at 17, 42, 57, and 72 °C.

and not the neighboring peaks can be shown by varying the magnesium ion concentration. As the magnesium concentration increases, L′ shifts upfield and B′ merges with B. At high magnesium concentrations where L′ is resolved, L′ gives an NOE to BB′ (Figure 5e). L′ is thus adjacent to B′. L′ does not appear to give NOEs to peaks other than B′.

Peak D appears to give effects at B as well as to the peak at 8.05 ppm. At high irradiation powers, when C and E are also affected by spillover, an additional NOE to peak N is seen (Figure 4e). Since peaks C and E do not directly affect N, this NOE is from peak D. An NOE from peak N to peak D has also been observed at low temperature although it is not apparent in Figure 5d because of the lability of peak D. Peak D is one of the first resonances to exchange on heating the tRNA (Figure 8). It therefore appears that peak N contains GC-30. Since N contains three resonances of which only two have been assigned, its NOEs remain ambiguous. NOEs from N are observed to peaks H (discussed above) I, and MM′ and more weakly to B (Figure 5d). The effect to MM′ is not from nonselective decoupling as, at this magnesium concentration, peak L′ is closer to N than is MM′, but there is a larger effect at MM′. Further evidence for the proximity of imino protons N and M is given by the melting studies discussed below. Either M, M′, or I could be GC-29. MM′ does not appear to give any NOEs, possibly because its neighbors are close in chemical shift. Peak I gives an NOE solely to peak N (Figure 5c).

(*B*) *CG Base Pairs and Their Neighbors.* Four single GC peaks remain unassigned, namely, F, I, J, and O, together with the multiple peaks EE′, KL, and MM′ and one component of peak N. Peaks I, N, and MM′ have been discussed above.

Irradiation of F gives effects at A and B (Figure 7c). These effects appear not to be due to irradiation spillover to peak G and are consistent with peak F being the m^{7}G-46 tertiary Hoogsteen hydrogen bond to CG-13, which is close in space to both base pair 14 (peak A) and base pair 12 (peak B). The 13.2 ppm shift of peak F at high magnesium is close to that of the m^{7}G-46 tertiary bond in *E. coli* tRNA^{Val} and yeast tRNA^{Phe} assigned at 13.35 ppm by chemical modification (Hurd & Reid, 1979b). Hence, F is assigned as the m^{7}G proton.

J gives NOEs to the complex peak DEE′F and to peak O at 37 °C (Figure 9c). At lower temperature the effect is to the partially resolved EE′ component of peak DEE′F. Thus E, J, and O are three consecutive GC base pairs. This assignment is confirmed by primary NOEs from peaks EE′ and O to J and by a secondary NOE from EE′ to O (Figures 7b and 9b). Peak O gives a small NOE to L′MM′ (Figure 9b), but this may be due to some irradiation spillover to the neighboring peak N, which gives a NOE to MM′; hence, the other neighbor of O is ambiguous. Peak EE′ gives an NOE to peak KL as well as to J (Figure 7b). That the NOE is to K or L and not to L′ can be shown by varying the temperature

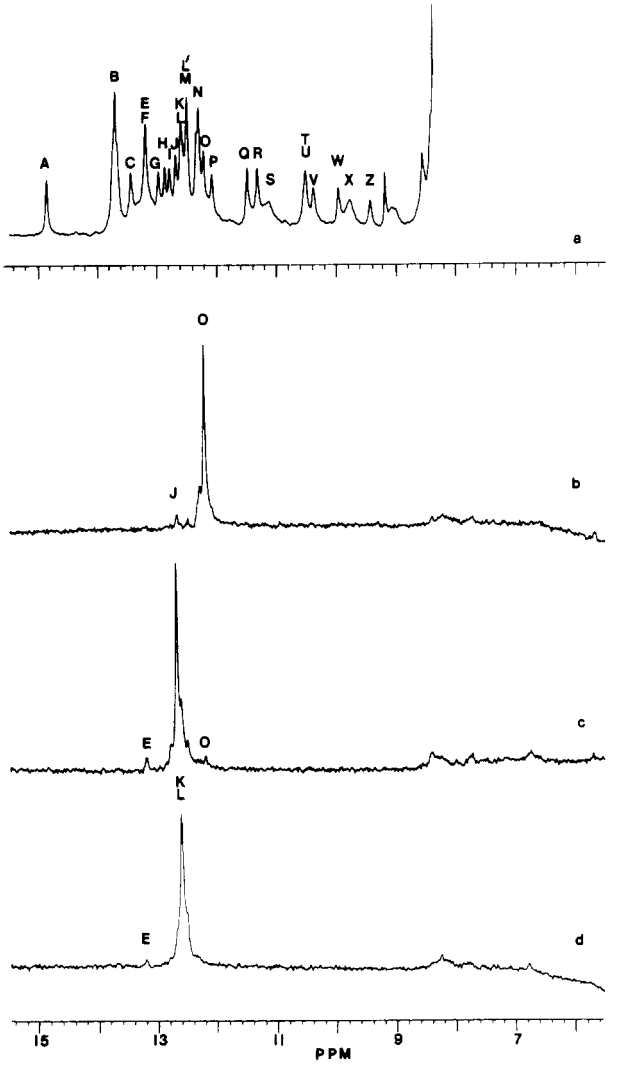

FIGURE 9: (a) Low-field ^1H NMR spectrum of *E. coli* $tRNA^{Phe}$ in 10 mM cacodylate–100 mM NaCl buffer, pH 7.0, containing 12 mM magnesium at 37 °C together with the NOE difference spectra obtained on irradiation of (b) peak O for 0.8 s, (c) peak J for 0.8 s, and (d) peak K for 0.8 s.

and magnesium concentration. The NOE to KL could be from either component of EE′; however, the chemical shift of EE′ at 13.1 ppm is close to the position of G-19-C-56 as assigned in yeast $tRNA^{Phe}$ (Johnston & Redfield, 1977) and in *E. coli* $tRNA_3^{Gly}$ (E. I. Hyde and B. R. Reid, unpublished observations). This Watson–Crick base pair is far removed from other base-paired imino protons in the X-ray crystal structure of yeast $tRNA^{Phe}$ (Kim et al., 1974) and so would not be expected to give NOEs. E′ is tentatively assigned to G-19-C-56, and K is assigned to a neighbor of E. Thus, KEJO are four consecutive GC base pairs. The double pair KL gives an NOE to EE′ but does not seem to give other effects (Figure 9d); hence, the other neighbors of KL are unknown. Both the anticodon and the acceptor stems of *E. coli* $tRNA^{Phe}$ contain more than three consecutive GC or CG base pairs, and so KEJO could be in either stem; however, two of the four GC base pairs in the anticodon stem (N and M or I) have been partially assigned, and hence, the KEJO string must be located somewhere in the acceptor stem.

Temperature Studies. The last stages of the imino resonance assignment in *E. coli* $tRNA^{Phe}$ are not possible purely by analysis of NOEs, due to the spectral overlap of the consecutive GC base pairs in the acceptor helix and the anticodon helix. Therefore, in order to help with the assignments of these

Table I: Experimentally Connected NOE Assignments of *E. coli* $tRNA^{Phe}$ and Comparison with Yeast $tRNA^{Phe}$

proton	assignment	position: *E. coli* $tRNA^{Phe}$ [a]	position: yeast $tRNA^{Phe}$
Ψ-55 N1-H	U	10.70	10.78[b]
Ψ-55 N3-H	Q	11.51	
T-54 A-58	B	13.75	
GC-53	H	12.83	
GC-52	N	12.30	
U-51	R	11.32	
G-63	W	10.06	
UA-50	B	13.75	
CG-49	C	13.48	
G-15-C-48	P	12.12	
m^7G-46-G-13	F	13.06	
s^4U-8-A-14	A	14.91	
CG-13	G	13.02	12.92[c]
UA-12	B	13.75	13.80[c]
CG-11			13.95[c]
GC-10			12.75[c]

[a] At 5 mM magnesium, 17 °C. [b] Tropp & Redfield, 1981. [c] Heerschap et al., 1982.

remaining resonances, spectra were measured at different temperatures (Figure 8). At high temperature, only six peaks remain in the ^1H NMR spectrum of *E. coli* $tRNA^{Phe}$; the other protons are in fast exchange with solvent and so are not observed. These same six resonances are left at high temperatures under all magnesium concentrations, but the melting temperature varies. By studying the melting behavior at different magnesium concentrations, it can be shown that these peaks are E or E′ or F, J, M, M′, N, and either N′ or O. Some ambiguities remain as peaks merge with temperature; however, since E′ and F have been assigned to tertiary base pairs that are not likely to survive at such high temperatures, it is probable that it is peak E that remains at high temperature together with the other neighbor of J, namely, O rather than N′.

These very stable resonances E, J, M, M′, N, and O have not been assigned above in the sequential ribothymidine and dihydrouridine stem NOEs and are therefore either from just the acceptor stem or from both the acceptor stem and the anticodon stem. In the acceptor stem there are six consecutive CG or GC base pairs, while in the anticodon stem there are four consecutive GC base pairs. In such runs of GC base pairs it is probable that the stems melt sequentially from the ends. The most stable resonances have been previously studied and their saturation–recovery rates at different temperatures measured (Hurd & Reid, 1980). The order of the saturation–recovery rates at high temperature are O > M′ > E > J > N = M. From the NOE data, peak J is located between base pairs E and O. As both E and O are more labile than J, if the base pairs in this helix melt sequentially, then the bases in the rest of this helix must be more labile than J, in fact more labile than E. However, N and M are in fact more stable than J and therefore must belong to a different helix from the E, J, and O resonances. Hence, we assign N and M to the anticodon stem. Since N and M are the least labile peaks in the spectrum, they are probably adjacent in the interior of this stem. This confirms the NOE between peaks N and MM′ and assigns M as GC-29. From its lability, M′ may be adjacent either to M or to E but not to O. Since K is already assigned adjacent to E, M′ is assigned to GC-28, by elimination.

DISCUSSION

Using the nuclear Overhauser effects and temperature effects detailed above, it is possible to assign the majority of the imino proton resonances of *E. coli* $tRNA^{Phe}$. The NOEs and

Table II: Imino Proton Assignments in *E. coli* tRNA^{Phe}

peak	position[a]	assignment	NOE partners
A	14.91	s^4U-8-A-14	G
B′	13.86	CG-11	L′
B	13.75	UA-12, T-54-A-58, UA-50, AU-7	G, H C, R, W, N
C	13.48	CG-49	B, R W,
D	13.37	AΨ-31	N
EE′	13.22	CG-2, G-19-C-56	J, KL
F	13.06	m^7G-46	A, B
G	13.02	CG-13	A, B
H	12.87	GC-53	B, N
I	12.82	GC-5	N
J	12.69	CG-3	E, O
K	12.63	GC-1	E
L	12.57	GC-27	
L′	12.63	GC-10	B′
MM′	12.51	GC-28, GC-29	N
N	12.34	GC-52, GC-30	B, H, I, N
	12.29	GC-5	
O	12.20	GC-4	J
P	12.12	G-15-C-48	A
Q	11.54	Ψ-55 N3-H	B, U, Z
R	11.35	U-51	B, N, W
S	11.35		
T	10.72		
U	10.72	Ψ-55 N1-H	Q
V	10.44		
W	10.06	G-63	B, N, R
XY	9.92		
Z	9.52	G-18-Ψ-55	B, Q

[a] At 5 mM magnesium, 17 °C.

assignments are summarized in Tables I and II.

The sequentially connected proton resonances of the ribothymidine and dihydrouridine stems are listed in Table I. Most of these protons give strong NOEs except for those between GC-52 and GU-51, where we have used the sequence information for assignment. In the ribothymidine stem, the ambiguity in NOEs from multiple peaks has been resolved by spin-diffusion to GC-53 and GC-52 from the thymine methyl group and by making use of the unique GU wobble base pair at position 51. The assignment of CG-49 as peak C rests on the weak secondary NOEs between peak C and the GU-51 protons RW because UA-50, the neighbor of GC-49, is part of a multiple peak (peak B). Further evidence for this assignment is given by the strength of the NOE between C and B. If the ribothymidine stem and acceptor helix are costacked in this tRNA as in the yeast tRNA^{Phe} crystal structure and in *E. coli* tRNA^{Ile} in solution (Hare & Reid, 1982b), CG-49 would be between two AU base pairs, AU-7 and AU-50, both probably in peak B (see below). Experimentally, peak C gives a very strong (double) NOE to B. Furthermore, peak C is also quite far downfield for a GC base pair, near the expected unshifted position, as predicted from the weak ring-current shifts from its neighbors (Arter & Schmidt, 1976). All of these facts support the assignment of C as GC-49.

As mentioned above, the NOE between the imino protons of base pairs GC-52 and GU-51 is weak, although that between AU-50 and GU-51 is strong while the intrabase pair NOE between the G and U imino protons is extremely strong, almost 90% after 1.0-s irradiation at 17 °C. Models of GU base-pair stacking show that GU base pairs stack better with Watson–Crick base pairs 5′ to the U than with those 3′ to the U (Mizuno & Sundaralingham, 1978), consistent with these data. Associated with this is the observation that the NOE from the thymidine methyl group to the GC-53 imino proton is weaker than that to the further GC-52 imino proton. This may indicate that GC-53 can relax more readily through its neighbors than can GC-52.

In the dihydrouridine stem only base pairs 14, 13, and 12 can be experimentally assigned by sequential NOEs. The assignment of CG-11 depends on the intensity of the complex peak B, which contains the resonances of the imino protons T-54-A-58 and UA-50 as well as UA12 and possibly one other proton. Integration of the areas of peaks BB′ under a variety of magnesium concentrations gives an intensity of between four and five resonances depending on how the unit intensity is chosen. Peak B′ is of unit intensity; therefore, peak B contains three or four resonances. Peak B gives NOEs to six resonances of which five have been assigned. If B contains three resonances, then the sixth NOE, to peak N, would be to CG-11 (peak I, a single resolved resonance that gives an NOE solely to peak N would tentatively be assigned to GC-10). However, if B contains four resonances, then the NOE to N could be from a proton other than UA-12. Comparison of the assignments of other tRNAs with the same D-stem sequence makes the assignment of N to CG-11 unlikely. Yeast tRNA^{Phe} and *E. coli* tRNA^{Val} and tRNA^{Lys} all have the same D-stem base sequence, and the shifts of their corresponding resonances are similar (Heerschap et al., 1982; Hare, 1983; Rajagopal and B. R. Reid, personal communication). The original report that CG-11 in tRNA^{Val} resonates at 12.3 ppm is an error resulting from the similar chemical shifts of UA-12 and CG-11 (D. R. Hare and B. R. Reid, unpublished results). Base pairs 14, 13, and 12 in *E. coli* tRNA^{Phe} have similar shifts to those in other tRNAs. In these tRNAs CG-11 resonates in the 13.6–13.8 ppm region (13.7 ppm in tRNA^{Val}), which is surprisingly deshielded for a GC pair. If this were the case in *E. coli* tRNA^{Phe}, CG-11 would resonate in peak BB′. Peak B or B′ may therefore contain CG-11. In view of the absence of an aromatic NOE from B′ at low magnesium concentrations, B′ is probably a GC pair and is tentatively assigned to CG-11; its neighbor, L′, then becomes GC-10.

In the anticodon stem four of the five base pairs have been assigned by a combination of NOEs and temperature effects. Peak D is assigned to AΨ-31 rather than AU-7 on the basis of its similarity in shift to AΨ-31 in yeast tRNA^{Phe} (Roy et al., 1982b). The NOE from peak D to peak B suggests that there is an AU-type base pair close to AΨ-31. In the yeast tRNA^{Phe} crystal structure bases 32 and 38 are stacked below AΨ-31 but cannot base pair because of their sequence. In *E. coli* tRNA^{Phe} these bases are Ψ and A, respectively. The NOE from D suggest that these are base paired and resonate in B. An analogous A-32-U-38 base pair has been observed in the ^{1}H NMR spectrum of *E. coli* tRNA$_2^{Ala}$ (Jones, 1982). There is no other place in the sequence of *E. coli* tRNA^{Phe} with two consecutive AU base pairs. The NOE from D to N shows that N contains GC-30, while the NOE and melting data suggest that peak MM′ contains GC-29 and GC-28.

In the acceptor stem, by elimination of all other AU base pairs, peak B or B′ contains AU-7. If B′ is assigned to CG-11, as above, then B contains AU-7, and the final NOE from B to N is to GC-6. The unassigned NOE from N to I is thus to GC-5. Peaks KEJO are then assigned to base pairs 1–4, probably in that order as K is more labile than O. This leaves the only unassigned peak in the hydrogen-bonded region, peak L, as GC-27.

The 9–12 ppm region of the spectrum is unusually rich in resolved resonances. The chemical shifts of these are indicative of atypically hydrogen-bonded or non-hydrogen-bonded imino protons. Five of these have been assigned above. Q and U have been assigned to Ψ-55 N3-H and Ψ-55 N1-H, respectively, R and W have been assigned the GU-51 wobble base pair, and Z has been assigned to G-18, which is probably

hydrogen bonded to Ψ-55 via its amino rather than its imino proton. The remaining peaks, S, T, V, X, and Y, and one resonance in peak R cannot be assigned rigorously in the present study as no NOEs to these peaks have been observed either from other imino protons or from the methyl groups of modified bases. Two of the peaks give NOEs to the aromatic region, V to a peak at 6.83 ppm and T to a peak at 6.92 ppm and, more weakly, to another peak at 7.5 ppm. The shifts of the protons at 6.8 and 6.9 ppm are indicative of pyrimidine C6-H resonances, and the NOEs are comparable in size to the NOE between Ψ-55 N1-H and Ψ-55 C6-H (Figure 3b). Primary NOEs to C6-H resonances can only occur from Ψ N1-H protons, and the only pseudouridine residues in *E. coli* $tRNA^{Phe}$ apart from Ψ-55 are at positions 32 and 40; thus, T and V may be from Ψ-32 N1-H and Ψ-40 N1-H. However, if one of these resonances is from Ψ-40 N1-H, AΨ-31 must be hydrogen bonded through Ψ N3-H in contrast to AΨ-31 in yeast $tRNA^{Phe}$, which is hydrogen bonded through Ψ N1-H (Roy et al., 1982b). Possibly one of the NOEs is second order.

The assignments of the peaks, including those made by inference, are given in Table II. The assignments are a necessary preliminary to any proton NMR study of the structure and interactions of this tRNA in solution. In the accompanying paper (Hyde & Reid, 1985), we study the effect of magnesium and spermine binding to this tRNA by using the above assignments to interpret the results.

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