Paramagnetic Ion Effects on the Nuclear Magnetic Resonance Spectrum of Transfer Ribonucleic Acid: Assignment of the 15–48 Tertiary Resonance†

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ABSTRACT: We have studied the effects of Co^{2+} and Mn^{2+} ions on the low-field nuclear magnetic resonance (NMR) spectra of pure class 1 transfer ribonucleic acid (tRNA) species. With 1.2 mM tRNA in the presence of 15 mM $MgCl_2$ discrete paramagnetic effects were observed for Co^{2+} at concentrations in the range 0.02–0.1 mM and for Mn^{2+} in the range 0.002–0.01 mM, indicating fast exchange of these cations with tRNA. Both of these cations paramagnetically relax the s^4U8–A14 resonance as well as other resonances from proximal base pairs. The Co^{2+} site appears to be the same site on G15 which was observed crystallographically [Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111*, 315–328]; the initially occupied tight Mn^{2+} site is the cation site involving the phosphate of U8. There are three base pairs within 10 Å of both sites, namely, G15–C48, A14–s^4U8, and C13–G22; this has led to the assignment of the G15–C48 and C13–G22 resonances in the NMR spectrum [Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111*, 315–328; Holbrook, S. R., Sussman, J. L., Warrant, R. W., Church, G. M., & Kim, Sung-Hou (1977) *Nucleic Acids Res. 4*, 2811–2820; Quigley, G. J., Teeter, M. M., & Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 64–68].

The low-field (−11 to −15 ppm) NMR[1] spectrum of a pure tRNA contains one proton per base pair and is potentially an extremely informative method of studying tRNA structure in solution provided the individual resonances can be assigned to their respective secondary and tertiary interactions [see Reid et al. (1979) and Hurd & Reid (1979)]. In our previous studies with several helical RNA hairpin fragments and intact tRNAs it is apparent that AU base pairs resonate upfield of −14.35 ppm and that GC base pairs resonate upfield of −13.45 ppm; the amount of upfield shift depends on the identity of the nearest neighbor nucleotides. Our previous NMR spectra are satisfactorily assigned by using the 11-fold helix neighboring shifts reported by Arter & Schmidt (1976) insofar as secondary base pairs in the interior of helixes are concerned.

However, secondary resonances from terminal base pairs, which may or may not be stacked on one side, remain problematical. A particular example is the case of C13–G22 at the end of the DHU helix in many tRNAs, including yeast $tRNA^{Phe}$; this resonance has been assigned at various positions in the spectrum between −13.1 and −11.5 ppm (Geerdes & Hilbers, 1977; Robillard et al., 1976; Kearns, 1976). The ambiguity derives from the uncertainty of the spatial orientation of A14 with respect to C13–G22.

In the crystal structure of yeast $tRNA^{Phe}$ (Quigley et al., 1975; Ladner et al., 1975) there are at least five tertiary interactions involving ring NH hydrogen bonds which contribute to the 7 ± 1 extra base pair resonances which we have observed in the solution structure of several related tRNAs (Reid et al., 1977). One of these interactions is the common G15–C48 tertiary base pair. At this position in the molecule the two interacting strands are parallel, and hence this tertiary GC pair is a reverse Watson–Crick interaction.

This atypical interaction has no precedent in model studies and poses a difficult assignment problem in the NMR spectrum. The G15–C48 interaction has been assigned at −13.5 ppm (Romer & Varadi, 1977), −11.7 ppm (Geerdes & Hilbers, 1977; Robillard et al., 1976), −12.9 ppm (Hilbers & Shulman, 1974), −10.0 or −9.5 ppm (Kearns, 1976), or −10.5 ppm (Bolton et al., 1976). While searching for possible ways to assign the resonance from the G15–C48 interaction, we became aware of the observation of Jack et al. (1977) that there is a Co^{2+} site in the crystal structure of yeast $tRNA^{Phe}$ in which the cobalt is directly coordinated to G15. In the tRNA crystal structure there are only three hydrogen-bonded ring NH protons within 10 Å of the paramagnetic cobalt ion, namely, G15–C48, C13–G22, and the 8–14 hydrogen bond. Thus, in the crystal structure these three protons should be paramagnetically affected by the bound Co^{2+}. In bacterial class 1 tRNAs the s^4U8–A14 tertiary resonance has been unambiguously assigned by three independent methods at −14.8 ±0.1 ppm by ourselves and others [see Hurd & Reid (1979)]. The exceptionally low-field position of this proton is due to the extremely deshielded N3H of s^4U. If the crystal structure predominates in solution and binding takes place at the crystallographic cobalt site, the −14.8-ppm s^4U8–A14 resonance, as well as the unassigned G15–C48 resonance and the ambiguous C13–G22 resonance, should be paramagnetically relaxed by low levels of Co^{2+}. In order to test this possibility, and hence to assign G15–C48 and C13–G22, we have investigated the effects of Co^{2+} additions on the NMR spectrum of several bacterial tRNAs. These tRNA species were chosen on the basis of their tertiary homology with yeast $tRNA^{Phe}$ in that they all contain U12–A23, C13–G22, A14 bonded to U8 or s^4U8, G15–C48, and A21 as the last residue of their DHU loop. Similar experiments have been carried out with Mn^{2+}; the results indicate that at very low levels this

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[1] Abbreviations used: tRNA, transfer ribonucleic acid; NMR, nuclear magnetic resonance; DHU, dihydrouridine.

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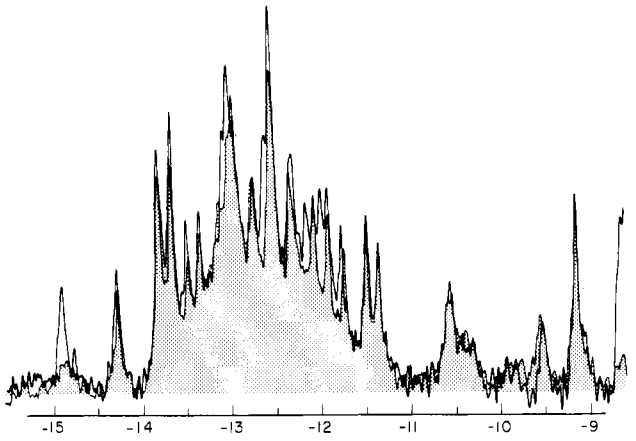

FIGURE 1: Effect of 0.1 mM $CoCl_2$ on the NMR spectrum of *E. coli* $tRNA_1^{Val}$ in the presence of excess magnesium ion. The Co^{2+} spectrum (stippled) has been overlayered on the spectrum in the absence of Co^{2+}; attempts have been made to scale the two spectra by accumulating the same number of sweeps at the same f1 power setting, but removing the sample for Co^{2+} addition and difficulties in replacing the tube in precisely the same position in the coil unavoidably cause small errors in exactly scaling the two spectra.

paramagnetic cation also binds at a site quite close to, but different from, the Co^{2+} site. The manganese effects corroborate the assignments derived from Co^{2+} binding.

Materials and Methods

tRNA Samples. The tRNA species used in this study were purified to homogeneity as described previously (Reid et al., 1977). All species were characterized by stoichiometry of amino acid acceptance (greater than 1700 pmol/A_{260}) and by two-dimensional RNase T1 fingerprints.

NMR Spectra. Six milligram aliquots of tRNA were dissolved in 0.19 mL of 10 mM sodium cacodylate (pH 7), 100 mM NaCl, and 15 mM $MgCl_2$ to give a final concentration of ~1.2 mM tRNA. Addition of Co^{2+} was accomplished by adding 2-μL aliquots of a 2 mM $CoCl_2$ solution to the tRNA solution, thus increasing the Co^{2+} concentration in the NMR microtube by 0.02 mM/addition. Spectra were obtained on a modified Bruker HXS-360 spectrometer at the Stanford Magnetic Resonance Laboratory by using fast-sweep correlation spectroscopy (Dadok & Sprecher, 1974). The sweep parameters were 2500 Hz in 0.8 s with a 0.2-s delay between sweeps; 1000 such transients were signal-averaged to give the final spectrum. Chemical shifts are expressed in parts per million from the reference DSS (2,2-dimethylsilapentane-5-sulfonate).

Mn^{2+} was added in 2-μL aliquots of a 0.25 mM solution of $MnCl_2$; thus, each addition raised the Mn^{2+} concentration by 0.0025 mM.

Results

Effect of Co^{2+} on the Escherichia coli $tRNA_1^{Val}$ Spectrum. The effect of 100 μM Co^{2+} on the low-field NMR spectrum of *E. coli* $tRNA_1^{Val}$ is shown in Figure 1. It is immediately obvious that the resonance from s^4U8–A14 (−14.9 ppm) is severely broadened in the presence of cobalt and that strong effects also occur at −12.05 and −12.25 ppm. There are also relaxation effects in the complex peak at −12.6 and −13.0 ppm, but problems in dilution, scaling the spectra, and possible partial broadening of several resonances in these multiple-proton peaks make this a less clear-cut observation. The fact that s^4U8 N3H (which is close to G15 in the crystal structure of tRNA) is seriously broadened suggests that, in solution, the Co^{2+} is occupying the crystallographic site in which it is coordinated to N7 of G15. Naturally, this interpretation depends upon the assumption that the tertiary folding of *E. coli* $tRNA_1^{Val}$ is analogous to that of yeast $tRNA^{Phe}$ determined crystallographically. Reassured by this observation, and the fact that G15–C48 and C13–G22 are also close to the cobalt site, we reasoned that these resonances should also be affected. Although the −12.05 and −12.25 ppm resonances appear qualitatively to be the most broadened, no clear distinction can be made at this stage between these two candidates and the broadened resonances in the complex peaks at −12.6 and −13.0 ppm.

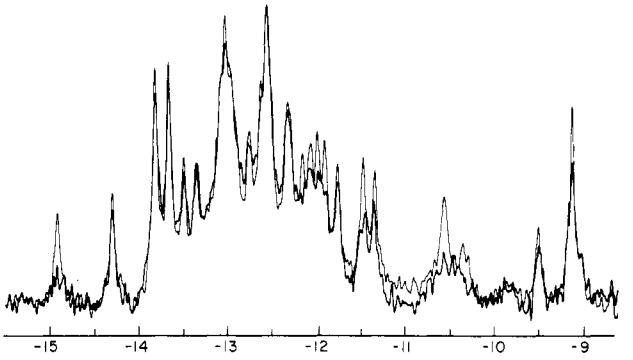

FIGURE 2: Effect of 0.0075 mM $MnCl_2$ on the NMR spectrum of *E. coli* $tRNA_1^{Val}$ in the presence of excess magnesium ion. The two spectra were directly plotted out on the same paper by using a slightly thinner pen for the spectrum in the absence of Mn^{2+}; the small errors in exactly scaling the spectra, discussed in Figure 1, also apply here.

Effect of Mn^{2+} on the E. coli $tRNA_1^{Val}$ Spectrum. The effect of 7.5 μM Mn^{2+} on the low-field NMR spectrum of *E. coli* $tRNA_1^{Val}$ is shown in Figure 2. Resonances at −14.9, −12.25, −12.05, −11.95, −11.5, and −10.5 ppm are severely broadened. Lesser broadening is observed for resonances at −11.4 and −9.1 ppm, and the complex peaks at −12.6 and −13.0 ppm are virtually unaffected. The severe broadening of the −14.9-ppm resonance indicates that Mn^{2+} binds within 10 Å of the s^4U8–A14 base pair. These effects are all detectable even at 0.25 μM Mn^{2+} and merely increase upon addition of further Mn^{2+}. Of the four magnesium sites which have been determined crystallographically (Holbrook et al., 1977; Jack et al., 1977; Quigley et al., 1978), only one is within 10 Å of U8 N3H; this is the site created by the closely juxtaposed phosphate 8 and phosphate 9 residues. Binding of manganese at this site is consistent with the effects observed in Figure 2. The relatively discrete nature of these effects, and the absence of marked effects on the 19 base pair resonances located between −14.3 and −12.4 ppm, argues against significant binding to the three other crystallographic magnesium sites at these low levels of manganese ion.

The *only* effects common to both Co^{2+} and Mn^{2+} are on the resonances at −14.9, −12.25, and −12.05 ppm. Since it is very likely that Mn^{2+} binds at the phosphate 8–phosphate 9 site, the only base pairs which lie within the sphere of the Co^{2+} effect and also within the sphere of the Mn^{2+} effect are s^4U8–A14, G15–C48, and CG13 (see Figure 3). Hence, as suggested qualitatively from the Co^{2+} data alone, G15–C48 and C13–G22 must be responsible for the resonances at −12.25 and −12.05 ppm.

Co^{2+} Effect on the E. coli $tRNA_m^{Met}$ Spectrum. The effect of 60 μM Co^{2+} on the low-field NMR spectrum of *E. coli* $tRNA_m^{Met}$ is shown in Figure 4. Again consistent with binding to G15, the s^4U8–A14 base pair resonance (−14.9 ppm) is broadened. Other severely broadened resonances are observed at −13.0 ppm and possibly at −12.9, −12.05, and −10 ppm. In addition, a shift of a resonance at −12.8 ppm upfield

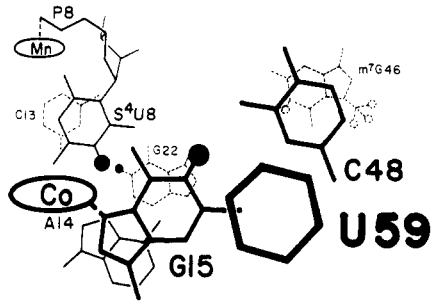

FIGURE 3: Diagrammatic view down the tRNA DHU helix axis showing the ring NH protons of 15–48, 8–14, and 13–22 (dark spheres) and the approximate location of the cobalt site and the P8 cation site described in the text (sketch by W.K.R. from a pair of stereo photographs of the $tRNA^{Phe}$ crystal structure kindly provided by Sung-Hou Kim; the Mn^{2+} site is actually slightly to the right, away from the viewer, bonded to P9 which has been omitted for clarity.

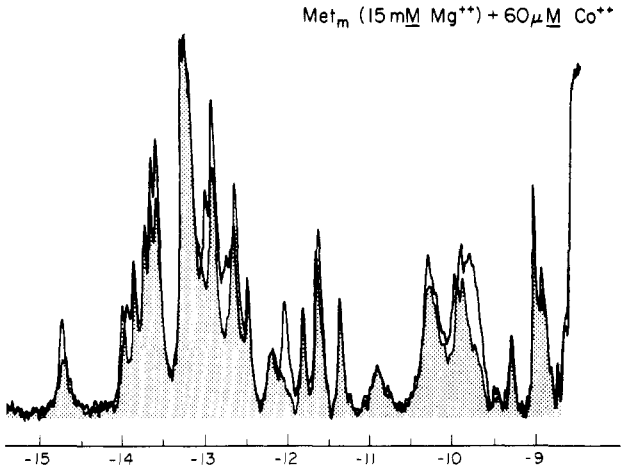

FIGURE 4: Effect of 0.06 mM $CoCl_2$ on the NMR spectrum of *E. coli* $tRNA_m^{Met}$ in the presence of excess magnesium ion. The Co^{2+} spectrum (stippled) is directly overlayered on the spectrum in the absence of Co^{2+}.

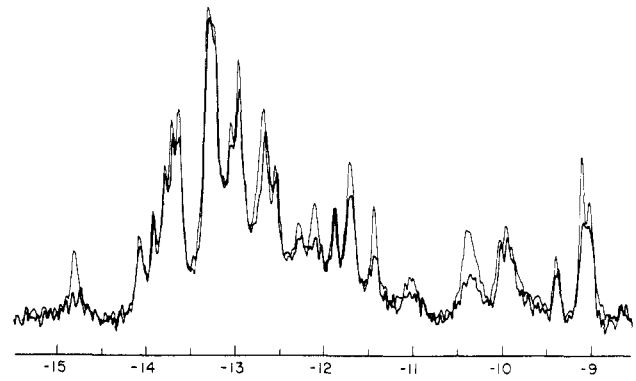

FIGURE 5: Effect of 0.005 mM $MnCl_2$ on the NMR spectrum of *E. coli* $tRNA_m^{Met}$ in the presence of excess magnesium ion. The Mn^{2+} spectrum (thicker line) was directly plotted on top of the spectrum in the absence of Mn^{2+} (slightly thinner line).

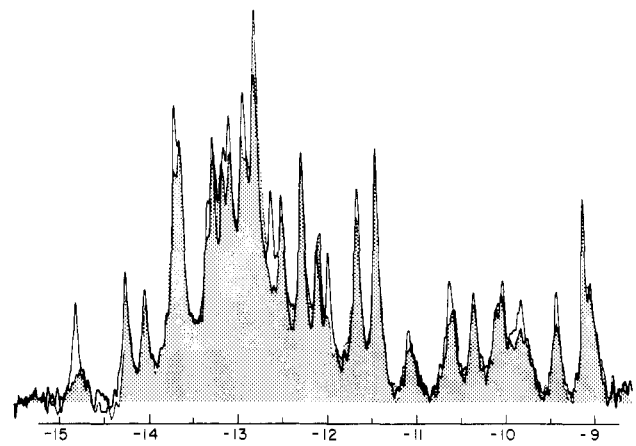

FIGURE 6: Effect of 0.01 mM $CoCl_2$ on the NMR spectrum of *E. coli* $tRNA^{Lys}$ in the presence of excess magnesium ion. The Co^{2+} spectrum (stippled) is plotted on top of the spectrum in the absence of Co^{2+}; there are probably small errors in exactly scaling the spectra as discussed in Figure 1.

to −12.7 ppm is observed, as well as a downfield shift of the proton at −14.0 ppm. The significance of these shifts is not clear.

The interesting feature of the $tRNA_m^{Met}$ structure is that while CG13 is in an identical environment to that in $tRNA_1^{Val}$, G15–C48 is not; G15–C48 has U59 stacked above it in $tRNA_1^{Val}$, whereas in $tRNA_m^{Met}$ U59 is replaced by A59. Thus, the resonance from C13–G22 (either −12.05 or −12.25 ppm in $tRNA_1^{Val}$) should remain at the same position in the spectrum of Met_m, whereas the G15–C48 resonance should move. Since only one resonance (at −12.05 ppm) is broadened in the region between −12.0 and −12.6 ppm in the $tRNA_m^{Met}$ spectrum, we assign it, and the −12.05-ppm resonance in the $tRNA_1^{Val}$ spectrum, to C13–G22. This leaves the resonances at −13.0, −12.9, and −12.8 ppm as candidates for the G15–C48 proton in $tRNA_m^{Met}$.

Effect of Mn^{2+} on the NMR Spectrum of E. coli $tRNA_m^{Met}$. Using the same logic as before, we should be able to identify the resonance from G15–C48 in $tRNA_m^{Met}$ as one of the resonances common to Mn^{2+} and Co^{2+} broadening. Figure 5 shows the effect of 5 μM Mn^{2+} on the low-field NMR spectrum of *E. coli* $tRNA_m^{Met}$. Strong broadening effects are observed for resonances at −14.9, −12.1, −11.5, −10.5, and −9.1 ppm. Resonances at −12.8, −12.35, and −11.8 ppm are affected to a lesser extent. As expected, resonances at −14.9 ppm (s^4U8–A14) and at −12.1 ppm (C13–G22) are strongly broadened. It is difficult, however, to find a third resonance which is affected by both Co^{2+} and Mn^{2+} broadening. The best candidate, the resonance at −12.8 ppm, appears only to shift with Co^{2+} in the *E. coli* $tRNA_m^{Met}$ spectrum. Thus, we can either assign G15–C48 at −12.8 ppm in the $tRNA_m^{Met}$ spectrum or consider the unlikely possibility that A59 might somehow disrupt the G15–C48 interaction in this molecule.

Effect of Co^{2+} on the E. coli $tRNA^{Lys}$ Spectrum. The ambiguous result of our attempts to assign G15–C48 in the $tRNA_m^{Met}$ spectrum led us to examine a second tRNA species for which U59 (in yeast $tRNA^{Phe}$, *E. coli* $tRNA_1^{Val}$) is replaced by A59. The effect of 100 μM Co^{2+} on the low-field NMR spectrum of *E. coli* $tRNA^{Lys}$ is shown in Figure 6. Again, as expected, a resonance at −14.9 ppm (s^4U8–A14) and a resonance at −12.05 ppm (C13–G22) are severely broadened; note that all species used in this series have the same DHU stem sequence so that the resonances from 14–8, 13–22, 12–23, etc. are expected in the same positions. The −12.05- and −14.9-ppm peaks are also broadened by Mn^{2+}, while other manganese-broadened peaks are not broadened by Co^{2+} (data not shown). However, a resonance at −12.6 ppm (broadened in the presence of 7.5 M Mn^{2+}) is shifted downfield to −12.8 ppm in the presence of 100 μM Co^{2+}. Thus, in both $tRNA_m^{Met}$ and $tRNA^{Lys}$ we find a resonance at ca. −12.7 ppm which is shifted by Co^{2+} and broadened by Mn^{2+}. Thus, it appears that we can tentatively assign G15–C48 at −12.7 ppm in $tRNA^{Lys}$ and at −12.8 ppm in $tRNA_m^{Met}$ (both species contain A59 rather than U59). In *E. coli* $tRNA_1^{Val}$ (and presumably in yeast $tRNA^{Phe}$ since they both contain U59) the G15–C48 resonance is at −12.25 ppm.

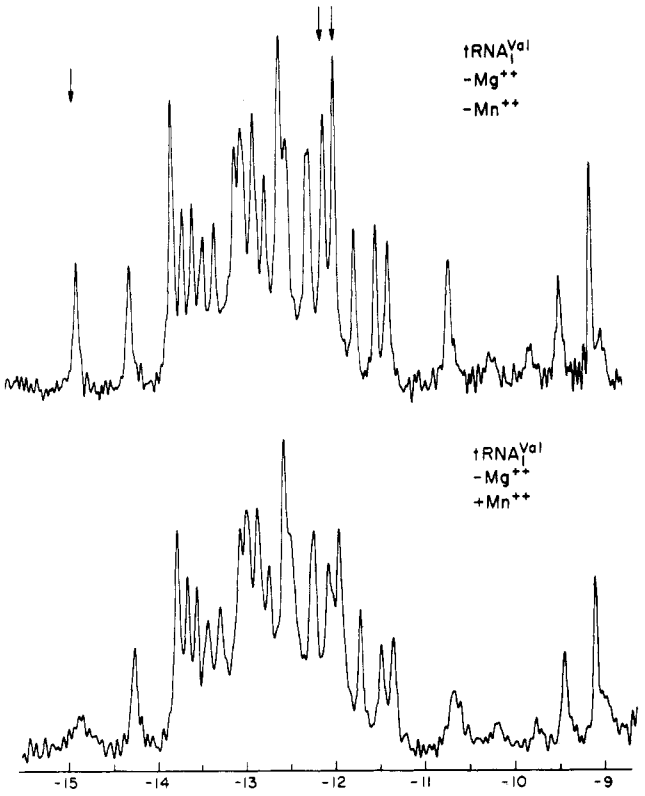

FIGURE 7: Paramagnetic effects of low levels of Mn^{2+} on the NMR spectrum of *E. coli* $tRNA_1^{Val}$ in the absence of magnesium ion. The upper spectrum is in 5 mM sodium phosphate, 200 mM NaCl, and 1 mM EDTA, pH 7.0. Addition of $MnCl_2$ to 0.8 mM produced no effects (presumably due to tight complexation by EDTA); further small additions to approximately 1 mM Mn^{2+} produced the observed effect (lower spectrum). The lower spectrum is slightly underscaled.

The paramagnetic studies discussed thus far were all carried out on tRNA in the presence of excess magnesium. We have also investigated the effect of Mn^{2+} on the low-field NMR spectrum of *E. coli* $tRNA_1^{Val}$ under high-salt conditions in the absence of magnesium. The high-salt, no-magnesium conformation of *E. coli* $tRNA_1^{Val}$ gives a better resolved spectrum below −12.5 ppm and a less well-resolved spectrum above −12.5 ppm than does the magnesium conformation (see Figures 1 and 7 in this manuscript and also the first paper of this series). Thus, while a positive result will not necessarily indicate that Mn^{2+} binds to this magnesium-deficient conformation (especially if the magnesium conformation is in fast exchange with the magnesium-deficient conformation), it does provide us with a method of resolving paramagnetic-affected resonances within complex peaks. The effect of Mn^{2+} on the low-field NMR spectrum of *E. coli* $tRNA_1^{Val}$ in 5 mM sodium phosphate, pH 7, 200 mM NaCl, and 1 mM EDTA is shown in Figure 7. The result is virtually identical with that observed when Mn^{2+} was added to *E. coli* $tRNA_1^{Val}$ in the presence of magnesium. The loss of resolution in the −12- to −12.4-ppm region (four single protons for the magnesium conformation and two peaks of intensity 2 for the no-magnesium conformation) makes it difficult, on the basis of these data alone, to determine whether two, three, or four resonances in that region are being affected by Mn^{2+}. The −12.0- and −10.5-ppm resonances, which are broadened by manganese in the magnesium spectrum, appear to be the only paramagnetically affected resonances which are found at a different chemical shift in the absence of magnesium.

Figure 8 shows the effect of a relatively high concentration of Co^{2+} (210 μM) on the low-field NMR spectrum of *E. coli* $tRNA_m^{Met}$. The interesting result is that more than 10 resonances remain sharp and unaffected by the Co^{2+}. This indicates that Co^{2+} is present at not more than one or two sites even at a Co^{2+}/tRNA ratio of 0.18.

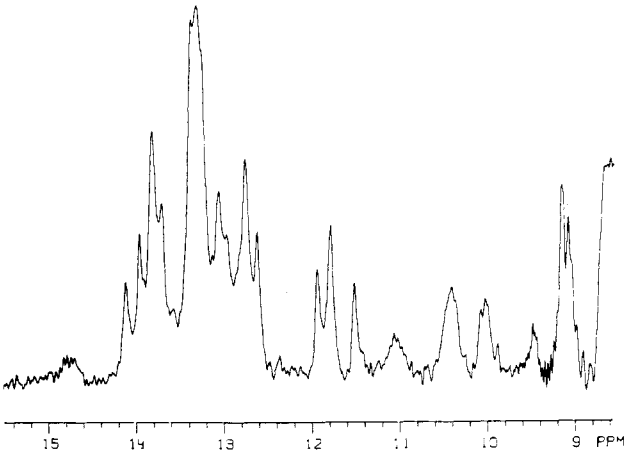

FIGURE 8: Effect of 0.21 mM $CoCl_2$ on the NMR spectrum of *E. coli* $tRNA_m^{Met}$ in the presence of excess magnesium ion. For a representative spectrum in the absence of any paramagnetic ions, see Figure 4.

Table I: Summary of Cobalt and Manganese Effects

	Val-1 Co^{2+}	Val-1 Mn^{2+}	Met-m Co^{2+}	Met-m Mn^{2+}	Lys Co^{2+}	Lys Mn^{2+}	Comments
*	14.9	14.9	14.8	14.8	14.8	14.8	s^4U8-A14
			14.0(s)			14.0	
	13.7	13.7	13.7	13.7	13.7	13.7	U12-A23 one or more
	13.5	13.5					weak
					13.4	13.4	weak
	13.1		13.1		13.1		one or more
			13.0		13.0		one or more
*			12.8(s)	12.8	12.7(s)	12.7	G15-C48 (A59)
	12.6						
*	12.3	12.3					G15-C48 (U59)
*	12.1	12.1	12.15	12.15	12.1	12.1	C13-G22
		12.0					
				11.8			
		11.5		11.5		11.5	
		10.5		10.5		10.5	U47 ?
		9.1		9.1		9.1	m^7G46 C8H

Discussion

Our interpretation of the comparative effects of paramagnetic ion binding to a series of tRNA species rests on the assumption that the structure of yeast $tRNA^{Phe}$ is a relevant model for the folding of $tRNA_1^{Val}$, $tRNA^{Lys}$, and $tRNA_m^{Met}$ from *E. coli*. The principal evidence in support of this assumption is the conservation of the residues involved in tertiary interactions: e.g., positions 8, 14, 15, 18, 19, 46, 48, 54, 55, 56, and 58 in the primary sequence of these related tRNAs. The effects of cobalt (at 0.015–0.08 Co^{2+}/tRNA) and manganese (at 0.002–0.006 Mn^{2+}/tRNA) on the low-field NMR spectra of *E. coli* $tRNA_1^{Val}$, $tRNA_m^{Met}$, and $tRNA^{Lys}$ are summarized in Table I. As described under Results, resonances from s^4U8–A14, C13–G22, and G15–C48 are the only ones common to the strong paramagnetic effects of both Co^{2+} and Mn^{2+} in all three tRNAs; these are indicated by asterisks. It is difficult to discern whether the additional, lesser effects listed are all from a single Co^{2+} site and a single Mn^{2+}

site or if these ions spend an appreciable amount of time at any other site or sites. One interesting observation is that only Mn^{2+} strongly broadens resonances at −11.5, −10.5, and −9.1 ppm in all three species and that only Co^{2+} strongly broadens one or more resonances at −13 ppm in all three species. At the present level of interpretation, however, it is difficult to use these data for assignment purposes since Co^{2+} and/or Mn^{2+} might conceivably bind at additional sites even with the low levels used. This uncertainty regarding possible secondary binding sites is more of a problem in attempts to interpret the weaker paramagnetic effects. For instance, a resonance at −13.7 ppm is weakly relaxed by both Co^{2+} and Mn^{2+} in the spectra of all three tRNA species; this resonance is probably from U12–A23, which is common to all three species and is reasonably close to both cation sites.

Chao & Kearns (1977) have investigated Mn^{2+} effects on the low-field and high-field (0–3 ppm) NMR spectra of unfractionated *E. coli* tRNA and yeast $tRNA^{Phe}$. They conclude from their unresolved spectra of crude tRNA that Mn^{2+} occupies three strong sites near s^4U8–A14, U33, and A58–T54 (in that order). In their unfractionated *E. coli* tRNA study their first titration gives a Mn^{2+}/tRNA ratio of 0.01; this is at least twice the Mn^{2+}/tRNA ratio at which we observe our initial broadening effects. When we increase the Mn^{2+}/tRNA to 0.01, we observe broadening of many additional resonances in pure tRNA species. Thus, at this cation stoichiometry we are almost certainly occupying multiple sites. In their studies on yeast $tRNA^{Phe}$, Chao & Kearns (1977) worked at Mn^{2+}/tRNA ratios similar to ours. They report broadening of resonances at −14.4, −11.5, and −10.5 ppm, but their spectra lack the resolution necessary to detect broadening in more complex peaks.

Although the paramagnetic effects we have presented here have been interpreted in light of the crystal structure, there are several independent conclusions which can be made without any previous knowledge or assumptions concerning the tertiary folding of tRNA. If we limit ourselves purely to solution data, we can deduce the following. (1) The resonance at −14.9 ppm is the N3H of s^4U at position 8. (2) The s^4U8 N3H, by virtue of its chemical shift and slow exchange with water, is hydrogen bonded to something. (3) In solution, s^4U8 is adjacent to CG13 since s^4U8 and C13 can be directly photo-cross-linked (Favre et al., 1969); here we assume that the cloverleaf is a valid representation of secondary pairing and that CG13 exists as a base pair. (4) The most reasonable acceptor for the s^4U8 hydrogen bond in the immediate environment of CG13 is the adjacent A14 residue, making an 8–14 pair. (5) Co^{2+} and Mn^{2+} bind close to the hydrogen-bonded s^4U8 (the 8–14 pair). (6) Since levels of these cations far below stoichiometric amounts relax *all* of the s^4U8 resonances in the population, Co^{2+} and Mn^{2+} are in fast exchange with tRNA. (7) The Co^{2+} site close to s^4U8 is *not* the same as the Mn^{2+} site close to s^4U8 (since the effects on the upfield resonances common to both cations are less than the effects at −14.9 ppm in the case of Mn^{2+}, whereas the reverse is true in the case of Co^{2+}). (8) The closest neighbors to the 8–14 pair in solution are CG13 and probably G15. (9) If G15 N1H is hydrogen bonded (with a reasonable helix lifetime), it will generate a low-field NMR peak. (10) Paramagnetic relaxation at s^4U8 will be accompanied by similar relaxation of the CG13 resonance and the G15 resonance (if G15 is hydrogen bonded). (11) Co^{2+}, bound close to s^4U8, also strongly relaxes the resonances at −12.25 and −12.05 ppm in *E. coli* $tRNA_1^{Val}$. (12) CG13 has identical sequence environments in $tRNA_1^{Val}$, $tRNA^{Lys}$, and $tRNA_m^{Met}$ and hence should have the same chemical shift in the spectra of these tRNAs; a resonance at −12.05 ppm in the spectra of all three tRNAs is relaxed by Co^{2+} and hence can be assigned to CG13. (13) The Co^{2+}-relaxed $tRNA_1^{Val}$ resonance at −12.25 ppm is probably derived from G15 hydrogen bonded to something.

These deductions can be made without any previous knowledge of tRNA tertiary folding or information on the crystallographic Co^{2+} site. Unfortunately, our solution experiments involving NMR reveal no information on the hydrogen-bond acceptor for G15. However, even before the X-ray analysis of tRNA, the probably existence of a hydrogen-bonded base pair from position 15 to position 48 was reported by Levitt (1969) based exclusively on an analysis of complementary coordinated base changes at these two positions. Thus, a combination of purely solution methods arrives at remarkably similar structural conclusions (at least in this region of the molecule) as those derived from X-ray structural studies. Thus, the data can be interpreted conversely as being consistent with the existence of the crystal structure in solution (although the fact that the crystals are grown from solution is good evidence in itself).

To summarize our solution data, the NMR experiments we have described lead to the assignment of the resonance from CG13 at −12.05 ppm and the resonance from G15–C48 at −12.25 ppm in the spectrum of *E. coli* $tRNA_1^{Val}$. The CG13 resonance remains at −12.05 to −12.1 ppm in $tRNA_m^{Met}$ and $tRNA^{Lys}$, but the G15–C48 resonance is shifted to ca. −12.75 ppm in these two tRNAs (presumably due to the presence of A59). As mentioned earlier, a proton from the G15–C48 tertiary interaction is generally accepted to exist somewhere in the tRNA low-field spectrum and has been "assigned" by several different laboratories at a variety of positions between −13.5 and −9.5 ppm. Assignment of this resonance at −13.5 ppm (Romer & Varadi, 1977) is inconsistent with the absence of broadening in this region by Mn^{2+} bound near s^4U8 (see Figure 2). Similarly, assignments of the 15–48 resonance between −9.5 and −11.8 ppm (Kearns, 1976; Geerdes & Hilbers, 1977; Bolton et al., 1976; Robillard et al., 1976) are also incorrect since no resonances in this region are paramagnetically relaxed by Co^{2+} bound to G15 (see Figure 1).

Leroy & Gueron have presented evidence that Mn^{2+} ions bind to *all* tRNA phosphates with similar affinities (the greatest affinity being no more than 5 times the average affinity in the case of yeast $tRNA^{Phe}$ at 30 °C). One way to reconcile our observations with their results would be that only in certain locations at the end of the extended D helix can the approach of the Mn^{2+} ion come close enough to relax *any* ring NH protons. Although this changes our assumptions about the cation site, it does not change our assignments.

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References

Arter, D. B., & Schmidt, P. G. (1976) *Nucleic Acids Res. 3*, 1437.

Bolton, P. H., Jones, C. R., Bastedo-Lerner, D., Wong, K. L., & Kearns, D. R. (1976) *Biochemistry 15*, 4370–4377.

Chao, Y. H., & Kearns, D. R. (1977) *Biochim. Biophys. Acta 477*, 20–27.

Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13*, 243.

Favre, A., Yaniv, M., & Michelson, A. M. (1969) *Biochem. Biophys. Res. Commun. 37*, 266.

Geerdes, H. A. M., & Hilbers, C. W. (1977) *Nucleic Acids Res. 4*, 207–221.
Hilbers, C. W., & Shulman, R. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3239–3242.
Holbrook, S. R., Sussman, J. L., Warrant, R. W., Church, G. M., & Kim, Sung-Hou (1977) *Nucleic Acid Res. 4*, 2811–2820.
Hurd, R. E., & Reid, B. R. (1979) *Biochemistry* (preceding paper in this issue).
Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111*, 315–328.
Kearns, D. R. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 18*, 91–149.
Ladner, J. E., Jack, A., Robertus, J. D., Brown, R. S., Rhodes, D., Clark, B. F. C., & Klug, A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4414–4418.
Levitt, M. (1969) *Nature (London) 224*, 759.
Leroy & Gueron (1978) *Proc. 7th Int. Conf. Magn. Reson. Biol. Systems*, Nara, Japan.
Quigley, G. J., Wang, A. H., Seeman, N. C., Suddath, F. L., Rich, A., Sussman, J. L., & Kim, S. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4866–4870.
Quigley, G. J., Teeter, M. M., & Rich, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 64–68.
Reid, B. R., Ribeiro, N. S., McCollum, L., Abbate, J., & Hurd, R. E. (1977) *Biochemistry 16*, 2086–2094.
Reid, B. R., McCollum, L., Ribeiro, N. S., Abbate, J., & Hurd, R. E. (1979) *Biochemistry* (first of four papers in this issue).
Robillard, G. T., Tarr, C. E., Vosman, F., & Berendsen, H. J. C. (1976) *Nature (London) 262*, 363–369.
Romer, R., & Varadi, V. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1561.

Nuclear Magnetic Resonance Studies on the Tertiary Folding of Transfer Ribonucleic Acid: Assignment of the 7-Methylguanosine Resonance†

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ABSTRACT: Analysis of the low-field nuclear magnetic resonance (NMR) spectra of several class 1 D4V5 transfer ribonucleic acid (tRNA) species containing 7-methylguanosine in their variable loops reveals a set of six to seven tertiary base pair resonances, one of which is always located at ca. −13.4 ppm. Other tRNA species which do not contain 7-methylguanosine do not contain the tertiary resonance at −13.4 ppm. Chemical removal of 7-methylguanosine from several tRNAs containing the same dihydrouridine (DHU) helix sequence as yeast tRNA^{Phe} results in the loss of the −13.4-ppm tertiary resonance. In the initiator methionine tRNA, which contains a different DHU helix sequence, the 7-methylguanosine hydrogen bond has been assigned at −14.55 ppm by chemical removal of this residue. In these experiments the aromatic C8H proton of 7-methylguanosine was also assigned (−9.1 ppm). The unexpectedly low-field position of the 7-methylguanosine resonance is explained by the deshielding effect of the delocalized positive charge in this nucleoside.

In a previous paper in this series (Reid et al., 1979) we have identified, by several methods, six or seven resonances in the low-field NMR[1] spectra of several class 1 tRNAs which are not derived from standard secondary base pairs. All of these transfer RNA species contain m^{7}G as the central nucleotide of a five-residue extra loop (Barrell & Clark, 1974). From the three-dimensional crystal structure of yeast tRNA^{Phe} (Kim et al., 1974a; Robertus et al., 1974; Sussman & Kim, 1976a,b; Quigley & Rich, 1976) it is evident that the ring nitrogen proton of m^{7}G46 is hydrogen bonded to N7 of guanosine-22 in the major groove of the DHU stem. The m^{7}G46–G22–C13 base triple (Figure 1) stabilizes the interaction of the DHU stem and the extra loop (G22–C13 is the terminal Watson–Crick pair in the DHU stem).

In this paper we establish that the crystallographically observed m^{7}G46–G22 tertiary interaction exists in solution for a series of class 1 (D4V5) tRNAs. Furthermore, we assign a specific resonance in the low-field (−15 to −11 ppm) proton NMR spectrum of each of these tRNAs to the hydrogen-bonded ring nitrogen proton of m^{7}G. Comparative studies on isoaccepting tRNA species which do not contain m^{7}G led to an initial tentative assignment of the m^{7}G hydrogen-bond resonance in those which do. This assignment was then confirmed by excision of m^{7}G from yeast tRNA^{Phe}, *Escherichia coli* tRNA$_1^{Val}$, *E. coli* tRNA$_m^{Met}$, and *E. coli* tRNA^{Lys}. These tRNA species have an identical DHU stem and hence should have a nearly identical environment surrounding the m^{7}G ring nitrogen proton. Further verification of our assignment was accomplished by removal of m^{7}G from *E. coli* tRNA$_f^{Met}$; the base pair sequence in the DHU stem of this tRNA differs from that of yeast tRNA^{Phe} and therefore changes the environment of the m^{7}G N1H hydrogen bond. The unexpectedly low-field position of the m^{7}G ring NH

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[1] Abbreviations used: NMR, nuclear magnetic resonance; tRNA, transfer ribonucleic acid; DHU, dihydrouridine; m^{7}G, N^7-methylguanosine; Ψ, pseudouridine.

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