# 5-Methylcytidine Is Required for Cooperative Binding of $Mg^{2+}$ and a Conformational Transition at the Anticodon Stem–Loop of Yeast Phenylalanine tRNA†

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ABSTRACT: The role of modified nucleosides in tRNA structure and ion binding has been investigated with chemically synthesized RNAs corresponding to the yeast $tRNA^{Phe}$ anticodon stem and loop ($tRNA^{Phe}_{AC}$). Incorporation of d($m^5C$) at position 14 of the stem of $tRNA^{Phe}_{AC}$-d($m^5C_{14}$), CCAGACUGAA-GAU-d($m^5C_{14}$)-UGG, analogous to $m^5C_{40}$ in native $tRNA^{Phe}$, introduced a strong $Mg^{2+}$ binding at a site distant from the $m^5C$. A $Mg^{2+}$-induced structural transition, detected by circular dichroism spectroscopy, was similar to that observed for the DNA analog of $tRNA^{Phe}_{AC}$ (Guenther et al., 1992; Dao et al., 1992). In contrast, $Mg^{2+}$ had little effect on unmodified $tRNA^{Phe}_{AC}$-r$C_{14}$ or $tRNA^{Phe}_{AC}$-d($C_{14}$). Modified $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) bound two $Mg^{2+}$ ions, and the binding was cooperative. The dissociation constant of the two $Mg^{2+}$ ions from $tRNA^{Phe}_{AC}$-d($m^5C_{14}$), $2.5 \times 10^{-9}$ $M^2$, is the result of an RNA structure significantly stabilized by $Mg^{2+}$ binding, $\Delta G = -11.7$ kcal/mol. The $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) structure, investigated by $^1H$ NMR, had a double stranded stem of five base pairs and two additional base pairs across what was a seven membered loop in the unmodified $tRNA^{Phe}_{AC}$. Methylation of cytidine in the yeast $tRNA^{Phe}_{AC}$ enables the molecule to form more than one conformation through a process regulated by $Mg^{2+}$ concentration. Thus, the simplest of posttranscriptional modifications of tRNA, a methylation, is involved in a somewhat distant, internal-site $Mg^{2+}$ binding and stabilization of tRNA structure, especially that of the anticodon stem and loop.

The amino acid accepting stem, the TΨC stem–loop, and the anticodon stem–loop components of tRNA can be relatively independent functional and structural domains of the tRNA's three dimensional structure. The amino acid accepting domains of *Escherichia coli* alanine, valine, and methionine tRNAs are good experimental substrates for their respective *E. coli* aminoacyl-tRNA synthetases (Francklyn & Schimmel, 1989; Shi & Schimmel, 1990; Schimmel, 1991; Frugier et al., 1992; Martinis & Schimmel, 1992). The unmodified TΨC stem and loop domains of *E. coli* $tRNA^{Val}$ and yeast $tRNA^{Phe}$ have been used successfully as substrates for the *E. coli* tRNA (uracil-5)-methyltransferase in studies of the enzyme's nucleoside recognition determinants (Gu & Santi, 1991, 1992). We have used DNA analogs of the yeast $tRNA^{Phe}$ anticodon stem and loop ($tDNA^{Phe}_{AC}$)[1] to investigate the importance of modified nucleosides and the 2′-OH to the nucleic acid's structure, dynamics, and $Mg^{2+}$ binding (Guenther et al., 1992; Dao et al., 1992). The X-ray-derived crystal structure of yeast $tRNA^{Phe}$ has four tightly bound $Mg^{2+}$ ions, one of which is located within the anticodon loop domain. Of the DNA analogs of the anticodon domain that were investigated, only $tDNA^{Phe}_{AC}$-d($U_{13}m^5C_{14}U_{15}$) exhibited a $Mg^{2+}$-induced conformational transition. The structural change was dependent on RNA-like modifications of the 3′ side of the stem, d($U_{13}$-$m^5C_{14}U_{15}$), corresponding to $\Psi_{39}m^5C_{40}U_{41}$ in native yeast $tRNA^{Phe}$. $Mg^{2+}$ binding by the DNA analog stabilized the base pairs of the stem and resulted in a two-base turn in the loop, instead of a seven-membered anticodon loop. However, a DNA analog of the yeast $tRNA^{Phe}$ anticodon stem and loop in which the stem was composed of deoxyribonucleosides, but without RNA-like modifications, was not able to bind 30S ribosomal subunits in the presence of poly(U) or poly[d(T)] (Koval'chuke et al., 1991). The tRNA anticodon stem and loop without modified nucleosides of the stem ($tRNA^{Phe}_{AC}$) did bind poly(U) programmed 30S ribosomal subunits, but not as well as the fully modified tRNA anticodon stem and loop, indicating that biological function was dependent on an RNA structure of the stem with modified nucleosides.

In order to analyze the structural importance of the modified nucleosides of the anticodon stem, we have synthesized both modified and unmodified $tRNA^{Phe}_{AC}$ sequences by automated chemical synthesis of the RNA. The $Mg^{2+}$ binding and structure of these molecules have been characterized by circular dichroism (CD) and NMR. A methylated cytidine, corresponding to $m^5C_{40}$ of the yeast $tRNA^{Phe}$ anticodon domain, was required for a tight binding of $Mg^{2+}$. The $Mg^{2+}$ binding induced a structural transition, stabilized existing base pairs and facilitated other base pairings, and altered base stacking and the backbone conformation of the tRNA anticodon stem and loop domain.

## MATERIALS AND METHODS

*Sample Preparation.* Three RNA oligoribonucleotides were synthesized by automated chemical synthesis using standard phosphoramidite chemistry (Usman et al., 1987). The RNA sequences, each 17 bases in length, corresponded

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[1] Abbreviations: CD, circular dichroism; $tRNA^{Phe}_{AC}$, the RNA sequence corresponding to the anticodon stem–loop of phenylalanine transfer RNA; $tDNA^{Phe}_{AC}$, the DNA analog of the yeast phenylalanine tRNA anticodon stem–loop.

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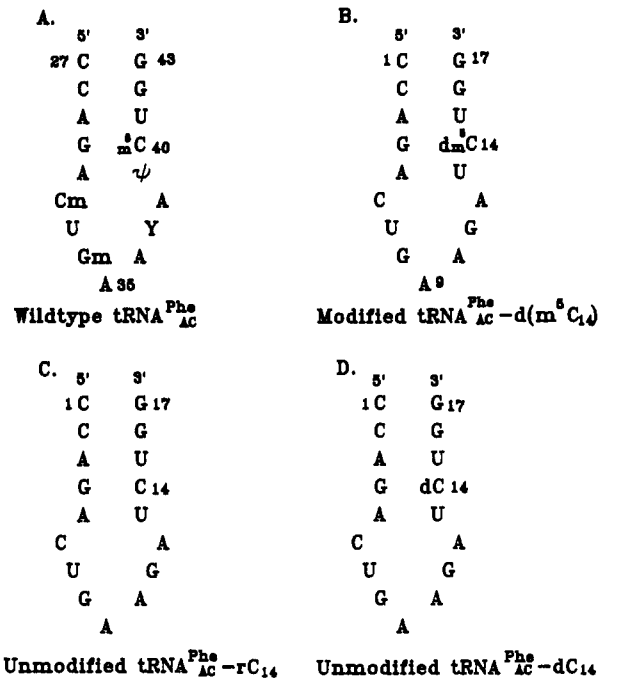


FIGURE 1: Sequences and secondary structures of the yeast tRNA$^{Phe}$ anticodon stem and loop and three chemically synthesized tRNA$^{Phe}_{AC}$ molecules. (A) The stem and loop formed by the anticodon arm of native yeast tRNA$^{Phe}$. Modified nucleotides include the 2′-*O*-methyl derivatives of C ($Cm_{32}$) and G ($Gm_{34}$), the hypermodified guanosine derivative wyosine ($Y_{37}$), pseudouridine ($\Psi_{39}$), and 5-methylcytidine ($m^5C_{40}$). (B) Modified tRNA$^{Phe}_{AC}$-d($m^5C_{14}$) with d($m^5C$) incorporated in position 14. (C) Unmodifed t-RNA$^{Phe}_{AC}$-r$C_{14}$. (D) Unmodified tRNA$^{Phe}_{AC}$-d($C_{14}$) with d(C) substitute for C at position 14.

to those of the anticodon stem and loop of yeast tRNA$^{Phe}$ (Figure 1). Two of the three oligomers, $C_1$CAGACUGA-AGAUC$_{14}$UGG and CCAGACUGAAGAU(dC)$_{14}$UGG, are unmodified, control molecules identified as unmodified tRNA$^{Phe}_{AC}$-r$C_{14}$ and tRNA$^{Phe}_{AC}$-d($C_{14}$). Modified tRNA$^{Phe}_{AC}$-d-($m^5C_{14}$) has a 5-methylated deoxycytidine incorporated in position 14, CCAGACUGAAGAU-d($m^5C_{14}$)-UGG, that corresponds to $m^5C$ at position 40 in the native tRNA$^{Phe}$ molecule. The RNAs were purified by HPLC, concentrated to a small volume, and precipitated with ethanol at −70 °C overnight (Guenther et al., 1992). The RNA pellet, collected by centrifugation, was dissolved in water. The nucleoside sequence of each RNA was confirmed by electrophoretic separation of fragments resulting from T1 RNase digestions. For CD and NMR spectroscopy the RNA solutions, in 10 mM sodium phosphate buffer at pH 7.0, were prepared as previously described for the DNA analogs (Guenther et al., 1992; Dao et al., 1992). The concentrations of the samples were measured and adjusted spectrophotometrically at 260 nm; the extinction coefficient was calculated with the nearest-neighbor approximation (Cantor et al., 1970). RNA solutions were titrated with the appropriate amounts of $MgCl_2$ as previously reported (Dao et al., 1992).

*Circular Dichroism (CD) Spectroscopy.* Circular dichroism (CD) spectra were recorded using a Jasco J600 spectropolarimeter and an interfaced IBM PC/2 microcomputer (Dao et al., 1992). Sample temperature was controlled with 1- or 0.1-cm-path-length, jacketed, cylindrical sample cells. All CD data were base line corrected for signals due to the cell and buffer. RNA spectra were independent of concentration to more than a 15-fold increase in RNA, indicating a lack of association or dimerization of the RNA oligomer. Even so, before addition of $Mg^{2+}$, all samples were heated at 70 °C for

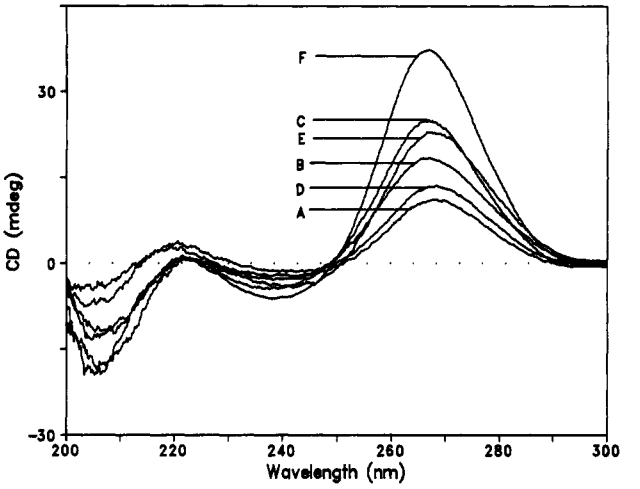


FIGURE 2: Comparison of CD spectra of unmodified tRNA$^{Phe}_{AC}$-r$C_{14}$ and tRNA$^{Phe}_{AC}$-d($C_{14}$) and modified tRNA$^{Phe}_{AC}$-d($m^5C_{14}$) in the presence and absence of $Mg^{2+}$. The CD spectra of 0.8 absorbance unit (260 nm) of the two unmodified tRNA$^{Phe}_{AC}$ molecules and t-RNA$^{Phe}_{AC}$-d($m^5C_{14}$) were collected at 10 °C in the presence and absence of 15 mM $Mg^{2+}$. (A) Unmodified tRNA$^{Phe}_{AC}$-r$C_{14}$ in the absence of $Mg^{2+}$. (B) Unmodified tRNA$^{Phe}_{AC}$-d($C_{14}$) in the absence of $Mg^{2+}$. (C) Modified tRNA$^{Phe}_{AC}$-d($m^5C_{14}$) in the absence of $Mg^{2+}$. (D) Unmodified tRNA$^{Phe}_{AC}$-r$C_{14}$ in the presence of $Mg^{2+}$. (E) Unmodified tRNA$^{Phe}_{AC}$-d($C_{14}$) in the presence of $Mg^{2+}$. (F) Modified tRNA$^{Phe}_{AC}$-d($m^5C_{14}$) in the presence of $Mg^{2+}$.

5 min and gradually cooled to room temperature in order to avoid any possible aggregation of the RNA molecules.

*NMR Experiments.* Proton NMR spectra were obtained at 500 MHz on a GE Omega instrument as previously reported (Guenther et al., 1992).

## RESULTS

*Differential Effect of $Mg^{2+}$ on the CD Spectra of Unmodified and Modified tRNA$^{Phe}_{AC}$.* We have reported that the CD spectra of unmodified tDNA$^{Phe}_{AC}$ and modified t-DNA$^{Phe}_{AC}$-d($U_{13}m^5C_{14}U_{15}$) were virtually identical in the absence of $Mg^{2+}$, but that the latter was profoundly affected by $Mg^{2+}$, while the former was not (Guenther et al., 1992). In order to investigate if a $m^5C$-dependent, $Mg^{2+}$-induced conformational transition occurred in tRNA, three yeast tRNA$^{Phe}_{AC}$ sequences (Figure 1B–D) were produced by automated chemical synthesis and are compared to that of native yeast tRNA$^{Phe}$ in Figure 1. Modified tRNA$^{Phe}_{AC}$-d-($m^5C_{14}$), B in Figure 1, has the same nucleoside sequence as that of native yeast tRNA$^{Phe}_{AC}$, but with only one modified nucleoside, d($m^5C_{14}$), located at the position analogous to that of $m^5C_{40}$ in the tRNA. Unmodified tRNA$^{Phe}_{AC}$-r$C_{14}$ (C in Figure 1) has no modified nucleoside, and tRNA$^{Phe}_{AC}$-d ($C_{14}$) (D in Figure 1) is a control on the placement of deoxyribose at position 14 of the anticodon stem. No changes in CD wavelength maxima and minima were observed with addition of 15 mM $Mg^{2+}$ to either unmodified t-RNA$^{Phe}_{AC}$-r$C_{14}$ or tRNA$^{Phe}_{AC}$-d($C_{14}$) or modified tRNA$^{Phe}_{AC}$-d($m^5C_{14}$) (Figure 2). However, the amplitude of the spectral wavelength maximum for modified tRNA$^{Phe}_{AC}$-d($m^5C_{14}$) increased 50%, whereas those of unmodified tRNA$^{Phe}_{AC}$-r$C_{14}$ and tRNA$^{Phe}_{AC}$-d($C_{14}$) were considerably less affected. The most obvious spectral differences occurred near 267 and 205 nm, regions of the spectrum indicative of base stacking and backbone conformation, respectively.

The differential effect of $Mg^{2+}$ on the CD spectra of modified tRNA$^{Phe}_{AC}$-d($m^5C_{14}$) versus unmodified tRNA$^{Phe}_{AC}$-r$C_{14}$

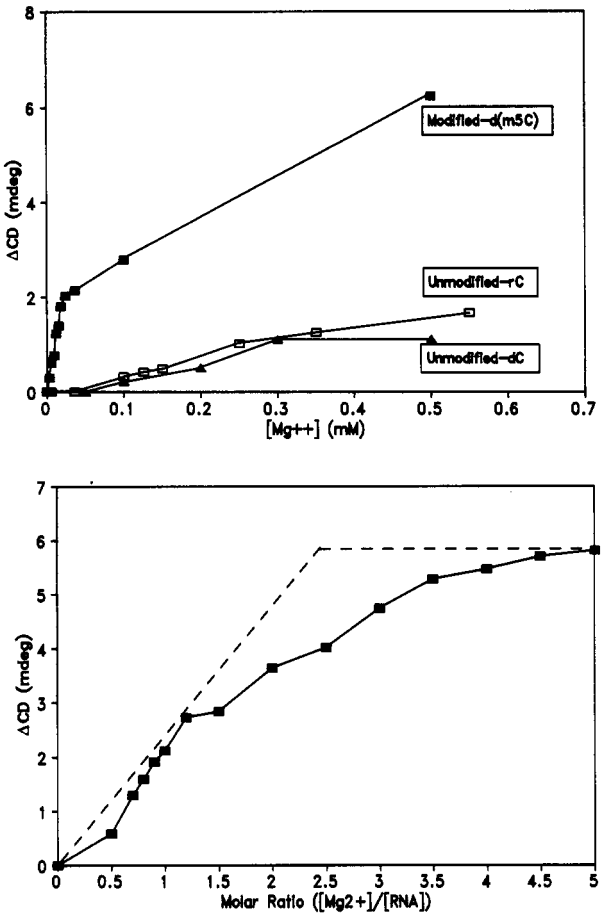


FIGURE 3: Titration of unmodified $tRNA_{AC}^{Phe}$ and modified t-$RNA_{AC}^{Phe}d(m^5C_{14})$ with $Mg^{2+}$. The CD spectra of modified t-$RNA_{AC}^{Phe}$-$d(m^5C_{14})$, unmodified $tRNA_{AC}^{Phe}$-$rC_{14}$, and $tRNA_{AC}^{Phe}$-d-$(C_{14})$ were collected at different $Mg^{2+}$ concentrations (micro- to millimolar). The change in the CD reading (Δmdeg) at maximum amplitude (267 nm) was monitored with increasing $Mg^{2+}$ concentration. (A) Modified $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$, unmodified $tRNA_{AC}^{Phe}$-$rC_{14}$, and $tRNA_{AC}^{Phe}$-$d(C_{14})$ at a concentration of 6 μM were titrated with $Mg^{2+}$ from 0 to 0.55 mM. (Not all data points are shown.) (B) A molar ratio titration was conducted of 6, 20, 50, and 100 μM modified $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ with various concentrations of $Mg^{2+}$. The titration of 100 μM $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ is shown. The change in CD (Δmdeg) is plotted against the ratio $[Mg^{2+}]$/[modified $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$].

and $tRNA_{AC}^{Phe}$-$d(C_{14})$ was investigated further by titrating each sample with $Mg^{2+}$. Structural changes were detected by monitoring changes in the CD spectra at a wavelength near the maxima for all three RNAs (Figure 3A). The CD spectra of unmodified $tRNA_{AC}^{Phe}$-$rC_{14}$ and $tRNA_{AC}^{Phe}$-$d(C_{14})$, monitored at 267 nm, remained unchanged until the $Mg^{2+}$ concentration reached 0.1 mM. Increases in $Mg^{2+}$ concentration beyond 0.1 mM produced only small incremental effects in the CD spectral maxima of the two unmodified molecules. Therefore, the nature of the ribose versus deoxyribose cytidine at position 14 was unimportant to the $Mg^{2+}$ binding. In contrast, the CD spectrum of modified $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ exhibited significant changes with addition of $Mg^{2+}$ at micromolar concentrations. The enhanced sensitivity of the CD spectrum of $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ to $Mg^{2+}$ indicated that methylation of $C_{14}$ strongly increased the $tRNA_{AC}^{Phe}$ domain's affinity for $Mg^{2+}$.

*Number of $Mg^{2+}$ Tightly Bound to $tRNA_{AC}^{Phe}$-$d(m^5C)$.* In order to determine the stoichiometry of the $Mg^{2+}$ binding to modified $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ in solution, a set of four molar ratio titrations were performed. $Mg^{2+}$ concentrations were increased to a maximum of 5-fold that of the $tRNA_{AC}^{Phe}$-d-$(m^5C_{14})$, which was titrated at concentrations of 6, 20, 50, and 100 μM. The 100 μM titration curve is shown in Figure 3B. The titration curve demonstrates that in the CD spectrum (ΔCD) changes of modified $tRNA_{AC}^{Phe}$, induced by $Mg^{2+}$ binding, approached completion when the $Mg^{2+}$:RNA molar ratio reached 5. A binding ratio of 2–2.5 $Mg^{2+}$ ions:1 modified $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ was obtained by extrapolating the initial slope of the titration curve to the maximum ΔCD obtained in titrating the RNA (Figure 3B). The curvature of a general molar ratio titration plot is determined by both the association constant, $K_a$, and the sample concentration; the curvature decreases with the increasing product $K_a$[sample] (Momoki et al., 1969). Thus, if the RNA concentration could be increased to a much higher level, the break point of the titration curve obtained by extrapolation might be shifted to a lower ratio. Therefore, a $Mg^{2+}$:$tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ binding ratio of 2 or below can be predicted from Figure 3B. On the other hand, increasing the RNA concentration by more than 15-fold did not affect the CD spectrum, indicating that aggregation of the molecule into dimers or trimers had not occurred. This result also eliminates the possibility of a $Mg^{2+}$: t-$RNA_{AC}^{Phe}$-$d(m^5C_{14})$ binding stoichiometry of 4:2, 2:2, etc. If we assume that only a single binding complex existed in solution, the results suggest that the $Mg^{2+}$ binds to modified $tRNA_{AC}^{Phe}$ with a stoichiometry of either 1:1 or 2:1.

A generalization of the molar-ratio method (Beltrán-Porter et al., 1983) was used to determine the stoichiometry and stability constant of $Mg^{2+}$ and modified $tRNA_{AC}^{Phe}$ binding. Briefly, in an equilibrium,

$$mA + nB \rightleftarrows A_mB_n$$

$$K_a = [A]^m[B]^n/[A_mB_n]$$

When the concentration of A, [A], is kept constant, a constant $K^*$ can be expressed as

$$K^* = (1/K_a[A]^{m-1})^{1/n} \quad (1)$$

$$= \{[B_x] - n[A](x)/m\}/\{(x)/m(1-x)^m\}^{1/n} \quad (2)$$

$[B_x]$ is defined as the ligand concentration that gives $x$ fraction of total equilibrium complex ($[A_mB_n]_{limit}=[A]/m$). In the titration experiment shown in Figure 3B, the concentration of modified $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ is kept constant at 100 μM. By setting $m = 1$ and $n = 1$ or 2, different $K^*$ values were calculated from eq 2 by using different values of $[Mg^{2+}]$ and $(x)$. The plot of $K^*$ vs $x$ is shown in Figure 4. Interestingly, a 2:1 binding ratio $Mg^{2+}$ to $tRNA_{AC}^{Phe}$-$d(m^5C_{14})$ was determined from the plot. The $K_d$ $(1/K_a)$ for the two $Mg^{2+}$ ions calculated from eq 1 is $2.5 \times 10^{-9}$ $M^2$. The free energy involved in the $Mg^{2+}$ stabilization of the RNA −11.7 kcal/mole, was determined with the equation $\Delta G^\circ = -RT \ln K_a$.

The 2:1 binding stoichiometry cannot be obtained directly by using Scatchard analysis because the concentration of free/bound $Mg^{2+}$ cannot be calculated from changes of CD spectra. However, the Scatchard analysis can be used to confirm the binding stoichiometry determined from the Beltrán-Porter method. The binding ratio obtained from the Scatchard analysis approximated 2.5. Furthermore, the nonlinear and convex shape of the Scatchard plot indicated that the binding is a strongly cooperative process according to McGhee's neighbor exclusion model (McGhee & Hippel, 1974).

*Modified $tRNA_{AC}^{Phe}$ Has a Closed Loop Structure.* The $m^5C$-dependent, $Mg^{2+}$-induced structural differences between modified and unmodified $tRNA_{AC}^{Phe}$-$rC_{14}$ were detected in

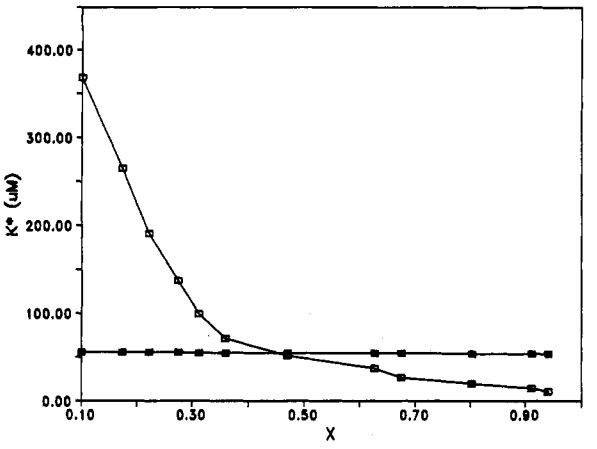


FIGURE 4: Determination of the stoichiometry of binding of $Mg^{2+}$ to modified $tRNA^{Phe}_{AC}$-d($m^5C_{14}$). Values of $K^*$ from eq 2 are plotted against $x$, the fraction of total $Mg^{2+}$–$tRNA^{Phe}_{AC}$ complex for different concentrations of $Mg^{2+}$. Results from the titration of 100 $\mu$M $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) with $Mg^{2+}$ and different values of $m$ and $n$ were used in eq 2 to calculate values of $K^*$. When the relative amounts of both $Mg^{2+}$ and $tRNA^{Phe}_{AC}$, the $n$ and $m$, respectively, of eq 2, are kept at 1, the curve shownwith open squares results. However, when the relative amount of $Mg^{2+}$ is 2 and that of $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) is 1, the line shown with closed squares results, indicating a $Mg^{2+}$:$tRNA^{Phe}_{AC}$-d($m^5C_{14}$) ratio of 2.

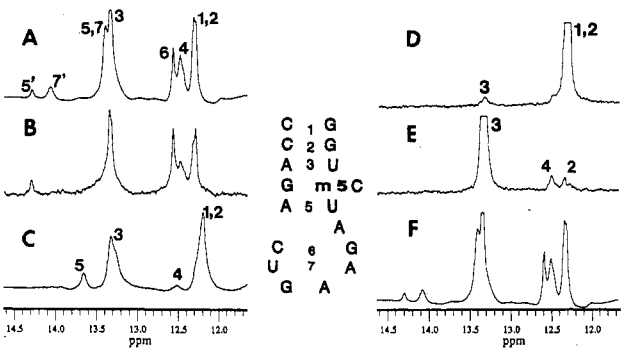


FIGURE 5: Proton NMR spectra of modified and unmodified $tRNA^{Phe}_{AC}$ in 5 mM $Mg^{2+}$ at 10 °C. NMR spectra of modified $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) (spectra A and F) and unmodified $tRNA^{Phe}_{AC}$ (spectrum C) in 5 mM $Mg^{2+}$ were obtained with the "1–1 hard pulse" in order to suppress the $H_2O$ resonance. The spectrum of modified $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) was also obtained by presaturation of the $H_2O$ resonance (spectrum B). The downfield portions of the three spectra are shown for analysis of the imino proton signals of the base pairs. Imino proton signals were assigned with the aid of NOE difference spectra such as the ones shown here demonstrating connectivities between $C_2$·$G_{16}$ and $A_3$·$U_{15}$ (spectrum D) and of $A_3$·$U_{15}$ to both $C_2$·$G_{16}$ and $G_4$·$m^5C_{14}$ (spectrum E). The numbered resonances of the spectra were assigned to the H-bonded protons of the base pairs shown in the diagram of the $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) stem and loop.

NMR spectra of the two molecules in the presence of 5 mM $Mg^{2+}$ at 10 °C. NMR spectra of modified $tRNA^{Phe}_{AC}$-d-($m^5C_{14}$) and unmodified $tRNA^{Phe}_{AC}$ were obtained with the "1–1 hard pulse" in order to suppress the $H_2O$ resonance. Imino proton signals were assigned with the aid of NOE difference spectra. All of the five potential base pairs in the stem of unmodified $tRNA^{Phe}_{AC}$-r$C_{14}$ were detected and assigned; no other H-bonded imino protons were detected (Figure 5). In contrast, the spectrum of the modified $tRNA^{Phe}_{AC}$-d-($m^5C_{14}$) exhibited two additional base pairs, numbered 6 and 7 in Figure 5. The two additional imino proton signals have been assigned to the $C_6$·$G_{11}$ and $U_7$·$A_{10}$ base pairs. Resonance 4, assigned to the $G_4$·$C_{14}$ base pair, was particularly weak for the unmodified $tRNA^{Phe}_{AC}$, indicating a difference in the stabilities of the two stems.

The base pairs of modified $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) in 5 mM $Mg^{2+}$ were not of equal stability. Imino proton exchange rates were different for various parts of the modified t-$RNA^{Phe}_{AC}$ structure, as exhibited by the differences in the spectra obtained with the 1–1 hard pulse versus presaturation methods. In comparing the 1–1 hard pulse and presaturation, signals 5 and 7, assigned to the A·U base pairs at the bottom of the stem and across the loop, respectively, had lost intensity relative to peaks 1, 2, and 3. Spectra of modified t-$RNA^{Phe}_{AC}$-d($m^5C^{14}$) exhibited two small NMR signals, designated 5′ and 7′, with NOE connectivities to $G_4$·$m^5C_{14}$ and $C_6$·$G_{11}$, indicating that perhaps a small amount of another conformation had been stabilized by the modification.

## DISCUSSION

The discovery of more than 60 modified nucleosides in the tRNAs of all organisms has stimulated interest in the roles they play in translation (Nishimura et al., 1983; Dirheimer et al., 1983; Björk et al., 1987; Gehrke et al., 1990; Agris, 1991; Björk, 1992). Base modifications located in and around the anticodon sequence of tRNA have an important role in modulation of translational efficiency and/or codon specificity (Björk et al., 1987; Agris, 1991; Björk, 1992). The roles of base modifications located in regions other than the anticodon were considered as stabilizing factors of the tRNA conformation, but the precise functions have not been well elucidated. In order to understand the biological functions, it is essential to investigate the relationships between individual and combined modifications and the structure of tRNA. Earlier studies have shown that unmodified T7 transcripts of yeast phenylalanine tRNA can be aminoacylated, but require higher $Mg^{2+}$ concentrations than native $tRNA^{Phe}$ (Sampson & Uhlenbeck, 1988; Hall et al., 1990; Hall & Sampson, 1989; Sampson et al., 1992). A structural study of this tRNA transcript by NMR showed that the transcript appeared to fold normally, and the spectral features of the transcript resembled those of native $tRNA^{Phe}$ only when the $Mg^{2+}$ concentration was very high (Hall et al., 1989). The site-selective, metal-catalyzed hydrolysis of T7 transcripts resulted in a fragmentation pattern different from that of the native $tRNA^{Phe}$ (Chow et al., 1992). $Mg^{2+}$ affected the fragment pattern of the unmodified molecule more than that of the native, particularly in the anticodon stem–loop. One of the four magnesium ions located in the X-ray derived crystal structure of yeast $tRNA^{Phe}$ was in the upper part of the anticodon loop (Quigley et al., 1978). These results indicated that modified nucleosides could be involved in $Mg^{2+}$ binding by tRNA and stabilization of tRNA structure, especially that of the anticodon stem and loop.

In the present study, the 17 nucleoside yeast $tRNA^{Phe}$ anticodon stem–loop was produced with and without modification by automated chemical synthesis. The synthesized RNA was used to investigate the role of modification in tRNA structure and ion binding. Incorporation of d($m^5C$) at the position analogous to $m^5C$ in native $tRNA^{Phe}$ introduced strong $Mg^{2+}$ binding at a site distant from the $m^5C$ and a $Mg^{2+}$-induced structural transition. Previously, we reported that the introduction of d($m^5C$) into the DNA analog of t-$RNA^{Phe}_{AC}$ produced a $Mg^{2+}$-induced conformational change (Guenther et al., 1992). CD spectra of unmodified t-$RNA^{Phe}_{AC}$-r$C_{14}$ and $tRNA^{Phe}_{AC}$-d($C_{14}$) were unaffected by micromolar $Mg^{2+}$, indicating that the $Mg^{2+}$-induced structural transitions in the anticodon stem–loop structures of t-$RNA^{Phe}_{AC}$-d($m^5C_{14}$) and $tDNA^{Phe}_{AC}$-d($U_{13}m^5C_{14}U_{15}$) were due to the methylation of cytidine and not the deoxyribose. The $tRNA^{Phe}_{AC}$-d($m^5C_{14}$) structure has a double-stranded stem of

five base pairs and two additional base pairs across what was a seven-membered loop, resulting in a two-base turn in the loop and presumably a single nucleoside bulge, $A_{12}$, on the 3′ side of the hairpin. The presence of $Mg^{2+}$ induced similar structural features in the $tDNA_{AC}^{Phe}$-d($U_{13}m^5C_{14}U_{15}$) (Guenther et al., 1992; Dao et al., 1992), indicating that the 2′-OHs were not necessarily required for ion coordination.

The binding ratio of $Mg^{2+}$ to modified $tRNA_{AC}^{Phe}$-d ($m^5C_{14}$) was calculated as 2:1, whereas only one $Mg^{2+}$ is found in the anticodon of the crystal structure of yeast $tRNA^{Phe}$ (Quigley et al., 1978) and in association with $tDNA_{AC}^{Phe}$ in solution (Dao et al., 1992). However, an examination of the ion binding sites in the crystal structure of yeast $tRNA^{Phe}$ reveals that there would be two potential ion binding sites in the chemically synthesized $tRNA_{AC}^{Phe}$ and its DNA analog. In the crystal structure, there is one $Mg^{2+}$ binding site located in the upper part of, and internal to, the anticodon loop. Another ion binding site, at which spermine binds the tRNA in crystal form, is located in the deep groove of the double helix formed by the D stem on top of the anticodon stem. The spermine appears to be hydrogen bonded to four different phosphate residues on both sides of the deep groove of the double helix (Schimmel & Redfield, 1980; Quigley et al., 1978). The synthesized $tRNA^{Phe}$ anticodon is composed of only half of this double helix; it lacks the D stem. One $Mg^{2+}$ is probably located internal to the anticodon loop, as it is in the crystal structure (Quigley et al., 1978) and the DNA analog in solution (Dao et al., 1992). The second $Mg^{2+}$ could be coordinated to two different phosphate residues in the "spermine binding site" of the RNA.

The strongly cooperative nature of the two $Mg^{2+}$ ions binding to the one modified $tRNA_{AC}^{Phe}$ molecule, as shown by Scatchard plot analysis, is consistent with results from studies of $Mg^{2+}$ binding to the entire tRNA molecule (Lynch & Schimmel, 1974). Equilibrium and kinetics studies utilizing a site-specific probe to follow structural changes in the native tRNA showed that the structural changes were associated specifically with cooperative ion binding (Lynch & Schimmel, 1974). Thus, cooperativity of ion binding is associated with the conformational changes needed to produce the native tertiary structure of tRNA. Therefore, we can predict that formation of the five base paired stem, the two additional base pairs across the loop, and the two-base turn of the modified $tRNA_{AC}^{Phe}$ is the result of both the presence of $m^5C$ and a cooperative $Mg^{2+}$ binding process.

The constant of dissociation of two $Mg^{2+}$ ions from $tRNA_{AC}^{Phe}$-d($m^5C_{14}$), 2.5 x $10^{-9}$ $M^2$, is considerably smaller than the constant calculated for the dissociation of one $Mg^{2+}$ ion from $tDNA_{AC}^{Phe}$-d($U_{13}m^5C_{14}U_{15}$), 1.09 × $10^{-6}$ M (Dao et al., 1992). The decreased dissociation constant is the result of an RNA structure more highly stabilized by $Mg^{2+}$ binding than the DNA analog, $\Delta G = -11.7$ versus $-7.75$ kcal/mol, respectively. Although the overall binding of $Mg^{2+}$ is stronger for the $tRNA_{AC}^{Phe}$, since there are two $Mg^{2+}$ ions bound, each $Mg^{2+}$ has a dissociation constant that is relatively larger than that of one $Mg^{2+}$ bound to the $tDNA_{AC}^{Phe}$ molecule. Therefore, the $m^5C$-dependent, $Mg^{2+}$-induced conformational transition of modified $tRNA^{Phe}$ provides a mechanism by which different microenvironments of $Mg^{2+}$ concentration could regulate the structure of the anticodon stem–loop domain in a manner not possible with the unmodified $tRNA_{AC}^{Phe}$.

Unmodified yeast $tRNA_{AC}^{Phe}$ binds poly(U)-programmed small ribosomal subunits with an affinity 2 orders of magnitude lower than the fully modified, native $tRNA_{AC}^{Phe}$ (Koval'chuke et al., 1991). The methylated cytidine at position 40 may be important for ribosome binding and other biological functions because it is required for the site-specific $Mg^{2+}$-regulated conformational change. Translational efficiency and specificity may be enhanced by the $m^5C$-dependent, $Mg^{2+}$-induced conformational transition. Aminoacyl-tRNA synthetase recognition of the tRNA and subsequent tRNA interaction with elongation factor and the ribosome could be differentially affected by the $Mg^{2+}$-regulated structural change (Dao et al., 1992).

## SUPPLEMENTARY MATERIAL AVAILABLE

Scatchard analysis of strong $Mg^{2+}$ binding to the modified $tRNA_{AC}^{Phe}$ (2 pages). Ordering information is given on any current masthead page.

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