High-Resolution Phosphorus Nuclear Magnetic Resonance Spectra of Yeast Phenylalanine Transfer Ribonucleic Acid. Metal Ion Effects and Tentative Partial Assignment of Signals†

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ABSTRACT: The temperature and metal ion dependence of the ^{31}P NMR spectra of yeast phenylalanine tRNA is presented, and a tentative assignment of some of the individual phosphate signals is offered. Signals A, C, F, P, and T have been assigned to the magnesium binding site phosphates by monitoring the magnesium ion dependence in the ^{31}P NMR spectra. In the presence of 10 mM Mg^{2+}, specific paramagnetic line broadening effects are observed for signals A, D, E, and U upon addition of 0.001–0.1 Mn^{2+} ion per tRNA molecule. Through the combination of the above metal ion experiments, X-ray studies, and earlier ^{1}H and ^{31}P NMR studies [particularly the tRNA modification ^{31}P NMR experiments of Salemink et al. [Salemink, P. J. M., Swarthof, T., & Hilbers, C. W. (1979) *Biochemistry 18*, 3477]], an initial assignment of some of these signals is attempted.

The ^{31}P NMR spectrum[1] of yeast phenylalanine tRNA ($tRNA^{Phe}$) has been shown to contain considerable fine structure. High-resolution ^{31}P NMR spectra (Gueron & Shulman, 1975; Gorenstein & Luxon, 1979; Salemink et al., 1979) revealed ~16 individual phosphate resonances spread over 7 ppm which were not observed in earlier ^{31}P spectra (Gueron, 1971; Weiner et al., 1974; Gorenstein & Kar, 1975).

We have proposed that ^{31}P chemical shifts of phosphate esters are sensitive to changes in O–P–O bond angles (Gorenstein, 1975) and ester torsional angles (Gorenstein & Kar, 1975; Gorenstein et al., 1976a; Gorenstein, 1977), and model system studies on single- and double-stranded nucleic acids (Gorenstein et al., 1976a; Gorenstein, 1978; Gorenstein & Luxon, 1979; D. G. Gorenstein, B. A. Luxon, J. B. Findlay, and E. M. Goldfield, unpublished results) suggested that a phosphate diester in a gauche, gauche (*g,g*) conformation should resonate several parts per million upfield from a diester in a nongauche conformation. It has been suggested, therefore, that stereoelectronic (bond and torsional angle) differences are responsible for the 7 ppm spread in the ^{31}P signals of the tRNA. An initial attempt to simulate the ^{31}P spectra of $tRNA^{Phe}$ based upon the X-ray crystallographically determined phosphate ester torsional angles supported the suggestion that the shifts in the scattered peaks are due to both torsional and bond angle distortions associated with tertiary structure (Gorenstein & Luxon, 1979). The observed coalescence of all of the diester signals at ~70 °C in the presence of 10 mM Mg^{2+} is consistent with the stereoelectronic origin for these ^{31}P shift differences since at this temperature the native structure melts into a random coil state.

Unfortunately, at the present time the theoretical basis for this stereoelectronic effect on the ^{31}P shifts is not reliable enough to allow us to accurately assign the scattered signals to individual phosphates. The precision of the $tRNA^{Phe}$ X-ray structure (Jack et al., 1976; Quigley et al., 1975; Stout et al., 1978; Sussman et al., 1978) is not sufficient anyway to accurately enough define the bond and torsional angles. In this paper we attempt to assign a number of these scattered signals from more traditional modification experiments. Specifically we have identified those signals which are sensitive to divalent metal ions (Mg^{2+} and Mn^{2+}). It has been shown by a number of experimental probes (Stein & Crothers, 1976; Schreier & Schimmel, 1974), including ^{1}H NMR (Bolton & Kearns, 1977a,b; Cohn et al., 1969; Chao & Kearns, 1977; Hurd et al., 1979) and X-ray diffraction (Hingerty et al., 1978; Holbrook et al., 1977; Quigley et al., 1978), that 4–5 divalent metal ions are tightly bound to tRNA. The cooperative interaction of these metal ions with the tRNA is essential for maintaining the native tertiary structure. Assignment of these metal ion binding sites is now quite firmly established and should help guide us in our assignment of the phosphate signals perturbed by divalent metal ions.

In conjunction with the yeast $tRNA^{Phe}$ modification and ^{31}P NMR experiments of Salemink et al. (1979) and the X-ray structural studies and ^{1}H NMR studies, we present an initial (and tentative) assignment of a number of the individual phosphate signals. Hopefully with further confirmation of these and additional signal assignments the full promise of phosphorus NMR in providing significant information on the solution structure and dynamics of these molecules will be realized.

Experimental Procedures

Yeast $tRNA^{Phe}$ was obtained from Boehringer Mannheim. Freshly boiled buffers and sterile glassware which had previously been leached for at least 1 h in 40% nitric acid were used.

Dialysis tubing was boiled for 1 h in 5% $NaHCO_3$ and 2% EDTA and then twice more in redistilled water for 1 h each to ensure removal of divalent metal ions. The tRNA (20 mg) was dissolved in 4 mL of glass redistilled water and dialyzed twice for at least 3 h at 4 °C against 1 L of buffer (0.1 M NaCl, 0.01 M cacodylate, and Mg^{2+} where indicated, pH 7.0) containing 10^{-2} M EDTA and then for 16–48 h at 4 °C against 1 L of buffer containing 10^{-3} M EDTA (one or two changes). The sample was concentrated on an Amicon high-pressure ultrafiltration cell using a UM-2 filter to 0.4 or 1.6 mL. For

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; CD, circular dichroism, EDTA, (ethylenedinitrilo)tetraacetic acid; tRNA, transfer ribonucleic acid.

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the high-field NMR studies the tRNA solution plus 20% D_2O for field-locking was placed in a Wilmad spherical microcell which in turn was inserted into a 12 mm or 20 mm o.d. NMR tube containing distilled water. In the low-field NMR studies the sample was placed in a 5-mm NMR tube.

High-field, Fourier transform ^{31}P NMR spectra were taken on Nicolet NTC-200 (80.9 MHz ^{31}P) or NTC-360 spectrometers (8.46 T; 145.7-MHz ^{31}P) with proton noise decoupling and 56° pulses, 4K data points, and 1.4-s recycle time. At low field, spectra were recorded on a Bruker WP-80 spectrometer at 32.4 MHz (^{31}P) with 70° pulses, 8K data points, and 2.05-s recycle times. The spectra were broad band ^1H decoupled. The temperature of the sample was controlled to within ±1 °C by Bruker or Nicolet temperature control units using nitrogen gas as a coolant. Decoupling at the superconducting fileds produced about 10–15 °C heating of the sample above the gas-steam measured temperatures, even when a gated two-level decoupling procedure [see Gorenstein & Luxon (1979)] was used.

In order to correct for the solution heating by the decoupler, a ^{31}P "thermometer" was designed. A solution of trimethyl phosphate (10 mM) and sodium hydrogen phosphate (10 mM) in a Tris buffer (100 mM) with 1 mM EDTA and in the appropriate salt solution (0.1 M NaCl) in 20% D_2O was adjusted to pH 7.0. The added salt was calculated to yield the approximate total ionic strength of the tRNA solution since decoupler heating is greater at higher salt concentrations. The frequency separation between the trimethyl phosphate and inorganic phosphate signals is temperature sensitive, and the shift range (1.5 ppm) and reproducibility of the measured shift difference from 0 to 90 °C is sufficient to calibrate the internal temperature of the ^{31}P thermometer to ±1 °C. The same "thermometer" sample was used on the NTC superconducting and Bruker WP-80 spectrometers. The temperature on the WP-80 spectrometer was directly measured in the probe with a Wilmad 5-mm thermometer. The ^{31}P shift difference (and hence sample temperature) on the Bruker spectrometer was the same with the decoupler off or set to 2.0 W. In contrast, the NTC-360 spectrometer decoupler heated the sample 4 (at 70 °C) to 16 °C (at 10 °C) even with the two-level decoupling procedure (gated low, 1 W at all times except during collection of the FID when it was gated high, 2 W). Single-level, 4-W decoupling produced greater than 25 °C heating and points out the importance of correction for this problem at very high fields. Decoupler heating was slightly less for the NTC-200 instrument.

All chemical shifts were referenced to 15% phosphoric acid in D_2O (0.00 ppm) at room temperature (25 °C). This sample is 0.453 ppm upfield from 85% H_3PO_4 with external D_2O lock. Positive chemical shfits are downfield from phosphoric acid.

tRNA spectral changes were reversible except that prolonged heating of some samples at $T > 60$ °C produced <1% nicks in the phosphodiester backbone. This was shown by the appearance of 2′,3′-cyclic nucleotide ^{31}P signals (Gorenstein & Kar, 1975) at ~20 ppm and additional phosphate monoester signals (3–4 ppm). All $tRNA^{Phe}$ samples originally had no nicks in the backbone. One of the $tRNA^{Phe}$ samples was, in fact, studied over a period of 4 months and was redialyzed and reconcentrated 4 times after numerous additions of aliquots of Mg^{2+} and Mn^{2+} solutions. The ^{31}P spectra under identical conditions were always unchanged, and no nicks were observed in this rather remarkable sample. One to ten microliter aliquots of a 2.48 mM $MgCl_2$ buffer solution (or Alfa ultrapure $Mg(NO_2)_2$) was added to the $tRNA^{Phe}$ solution. Manganese chloride was added in 2–10-μL aliquots of a 0.25 mM solution. In order to avoid hydrolysis of the tRNA samples it is important to readjust the pH of the tRNA solutions with very slow addition of 0.1 M NaOH at 4 °C with rapid stirring.

Results

^{31}P NMR Spectra of Yeast $tRNA^{Phe}$ in 10 mM Mg^{2+}. The ^{31}P NMR spectrum of yeast $tRNA^{Phe}$ in 10 mM magnesium ion buffers at various temperatures is shown in Figure 1. As Gueron & Shulman (1975), Gorenstein & Luxon (1979), and Salemink et al. (1979) have earlier shown for the ^{31}P NMR spectrum of yeast $tRNA^{Phe}$ between +3.1 and +3.4 ppm (peak A) is the 3′-terminal phosphate which integrates for a single phosphate residue and is the only signal which is pH sensitive (being a monoester with $pK \sim 6$). Between 0 and −1.5 ppm is a main cluster of signals representing the undistorted phosphate diesters in the double helical stems and hairpin loops (see Discussion). This main cluster integrates for ~59 phosphates. Upfield and downfield of the main cluster, spread over 6–7 ppm, are ~16 scattered signals, of which a number are well resolved at 30 °C. As shown in Figure 1 other scattered signals become better resolved at different temperatures. Individually resolved signals (such as B, C, D, T, and U) integrate for ~1 phosphate.

The temperature dependence of the ^{31}P chemical shifts of the labeled signals in Figure 1 is shown in Figure 2. Between 20 and 60 °C most of the scattered and main cluster signals shift very little with temperature. As shown earlier (Gorenstein et al., 1976; Gorenstein, 1978, 1981; Gorenstein & Luxon, 1979) this temperature insensitivity to most features in the tRNA ^{31}P spectra in 10 mM Mg^{2+} suggests that the tRNA (and the backbone phosphates) retains its native conformation throughout this temperature range. Eventually at $T > T_m \sim 70$ °C, all of the diester peaks merge into a single signal with the tRNA melting into a random coil conformation.

Although the major cluster signals and most of the scattered peaks show only small shifts with temperature, several interesting exceptions are notable (see Figure 2). Peak C shifts 0.55 ppm upfield from 22 to 50 °C while peak E shifts 0.4 ppm downfield. The two peaks and peak D (which shifts little) merge at ~50 °C, and between 40 and 46 °C the integrated intensity for the peaks decreases by 0.75 phosphate. At higher temperature a new broad upfield peak (integrating for ~1 phosphate) shifts upfield away from the merged signals. Although it is not possible to definitely establish which of the three peaks disappears and which shifts further upfield, on the assumption that the shift trends are continuous over the entire temperature range, it appears that C shifts ca. 1 ppm upfield and broadens while E shifts 0.6 ppm downfield with increasing temperature. D shifts very little over this temperature range and does not broaden. Peaks F, P, T, and U are the only other peaks which display a large shift with temperature, shifting 0.3–1.7 ppm.

Mg^{2+} Dependence to $tRNA^{Phe}$ ^{31}P Spectra. At lower temperature ($T < 40$ °C) the ^{31}P NMR spectra of $tRNA^{Phe}$ in the absence or in the presence of 10 mM Mg^{2+} are basically quite similar, as also previously concluded by Gueron & Shulman (1975), Gorenstein & Luxon (1979) and D. G. Gorenstein et al. (unpublished results). Apparently even without Mg^{2+} but in ~0.2 M total Na^+ (0.1 M added NaCl) the native secondary and tertiary structure is similarly stabilized at lower temperatures. However, as shown in Figures 3–6, a number of differences do exist even at 19 °C. By titrating the $tRNA^{Phe}$ solution with added Mg^{2+}, it is possible to identify which signals in the absence of Mg^{2+} correspond to the signals in the presence of Mg^{2+}, and these signals are

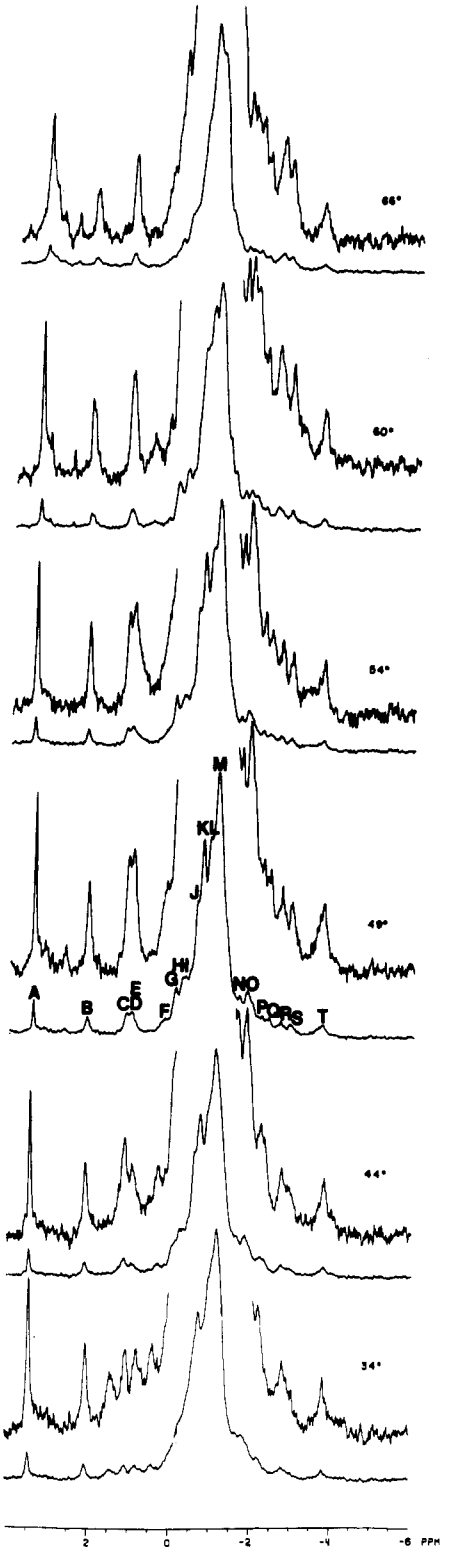

FIGURE 1: ^{31}P NMR spectra of yeast phenylalanine tRNA (~33 mg/mL) in 100 mM NaCl, 10 mM cacodylate, 10 mM $MgCl_2$, 1 mM EDTA, and 10% D_2O, pH 7.0, at indicated temperatures (°C), 145.8 MHz. The expanded scale for the scattered peaks is shown over the normal spectrum; 2-Hz line broadening applied to 8000 FID's.

labeled in Figures 5 and 6. This comparison shows that at 39 °C in the absence of Mg^{2+}, peak C is shifted 0.6 ppm upfield while peaks J, K, L, and M are shifted 0.2 ppm upfield upon addition of 30 mM Mg^{2+}. Peaks N and T are shifted 0.3 ppm upfield, and other diester signals experience only small shifts (monoester signal A shifts 0.4 ppm downfield). Addition of very high concentrations of Mg^{2+} (20–30 mM) essentially eliminates the temperature dependence to the ^{31}P chemical

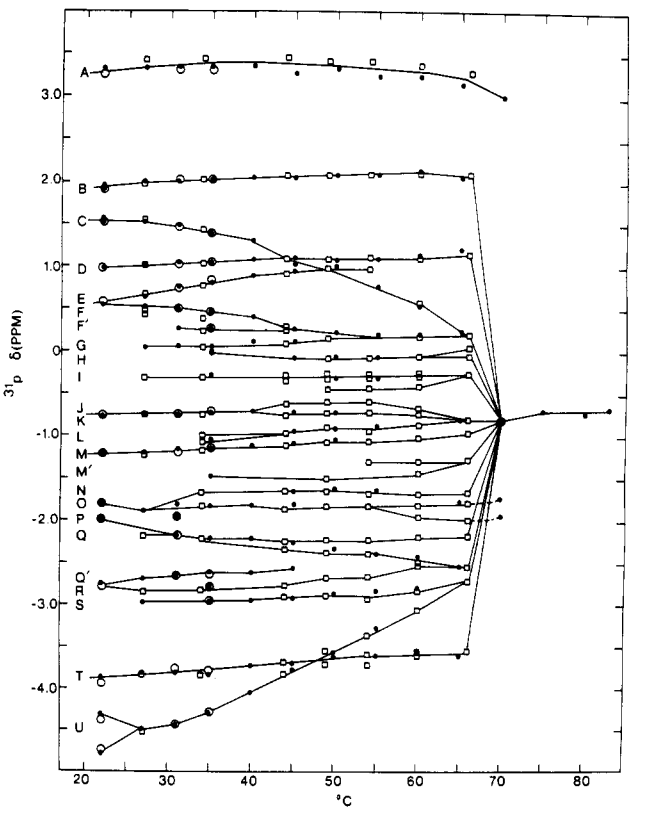

FIGURE 2: Temperature dependence of the ^{31}P chemical shifts, δ, of the peaks identified in Figure 1 for $tRNA^{Phe}$ in 10 mM Mg^{2+} buffer: (□) 145.8 MHz; (●) 32.37 MHz; (○) repeat spectra at 32.37 MHz.

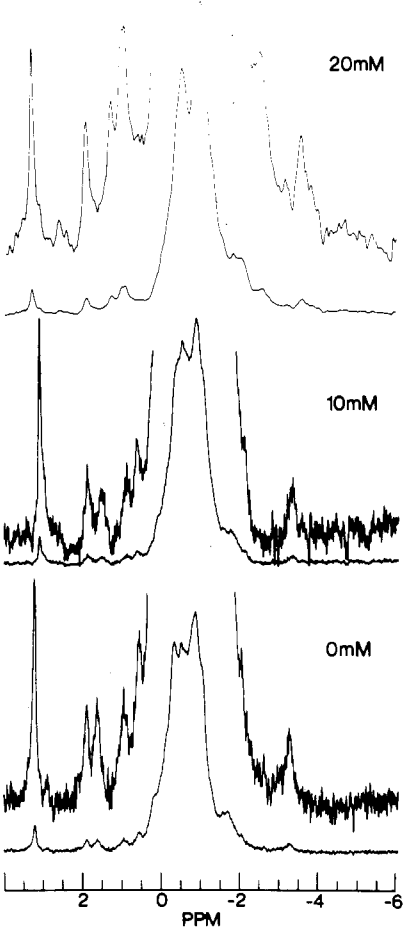

FIGURE 3: ^{31}P NMR spectra of $tRNA^{Phe}$ in standard buffer solution, 19 °C, and various $MgCl_2$ concentrations at 145.8 MHz (0 and 10 mM $MgCl_2$) and 32.4 MHz (20 mM $MgCl_2$).

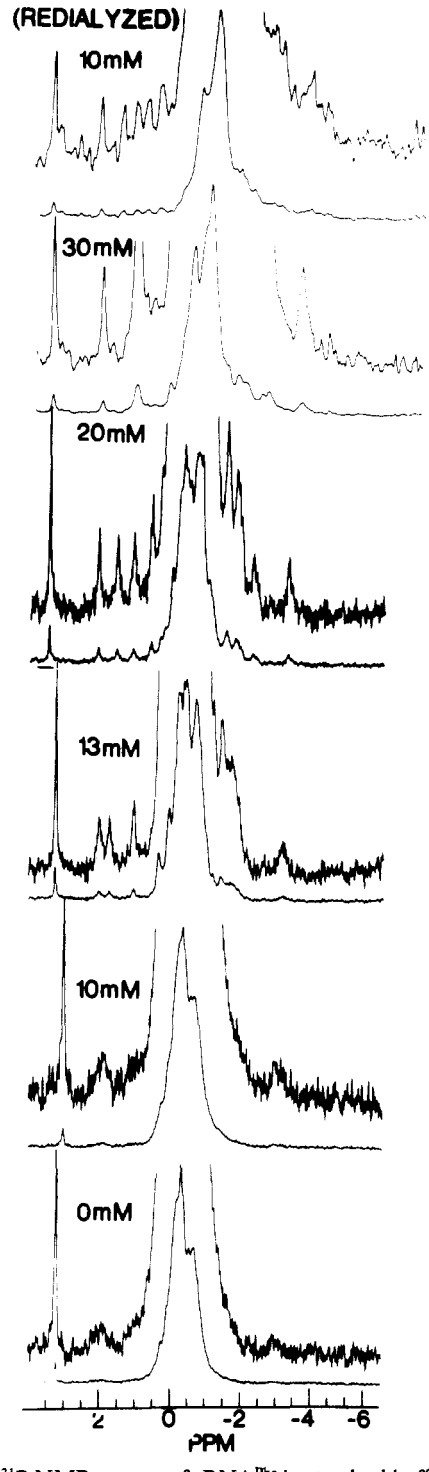

FIGURE 4: ^{31}P NMR spectra of $tRNA^{Phe}$ in standard buffer solution, 39 °C, and various $MgCl_2$ concentrations at 145.8 MHz (0, 10, 13, and 20 mM $MgCl_2$) and 32.4 MHz (30 mM and redialyzed 10 mM $MgCl_2$); 2-Hz line broadening applied to FID's.

shifts of the scattered peaks C, E, F, and P (Figure 7). These changes were found to be reversible: the same sample was originally dialyzed against buffer with no added Mg^{2+} and subsequently adjusted to 30 mM Mg^{2+}. This sample was then redialyzed against 10 mM Mg^{2+} and the ^{31}P NMR spectra shown to be identical with that of a sample which had been dialyzed only against 10 mM Mg^{2+} buffer. No nicks in the sugar–phosphate backbone as shown by the absence of 2′,3′-cyclic or additional phosphate monoester signals were observed during this treatment extending over several months.

In addition the integrated intensity for the scattered peaks (B–F; N–O) is only ~3 in the absence of Mg^{2+} compared to

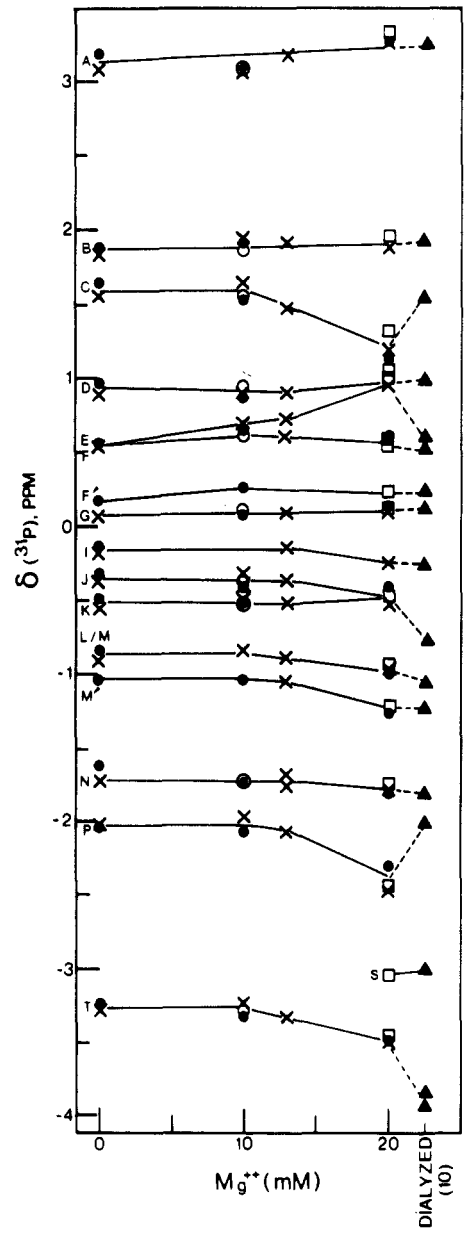

FIGURE 5: Magnesium dependence of the ^{31}P chemical shifts, δ, of the $tRNA^{Phe}$ peaks shown in Figure 3, 19 °C. Sample 1, 145.8 MHz (●); sample 2, 145.8 MHz (○); sample 1, 32.4 MHz (□); sample 1 redialyzed against 10 mM $MgCl_2$, 32.4 MHz (▲).

14 at 39 °C in 30 mM Mg^{2+}. At 19 °C, peaks B–E and N–U integrate for a total of 9 phosphates and 14 phosphates at 0 and 20 mM Mg^{2+}, respectively (Figure 8). As shown in Figures 8 and 9 the extra intensity at low Mg^{2+} concentrations appears in the spectral region which includes peaks G–K.

Mn^{2+} Effects on ^{31}P Spectra of $tRNA^{Phe}$. Addition of up to 0.15 mM Mn^{2+} (0.12 Mn^{2+} per yeast $tRNA^{Phe}$ molecule) in 10 mM Mg^{2+}, 0.1 M NaCl, 10 mM cacodylate, *no* EDTA, 32 °C, produces negligible chemical shift (<0.1 ppm) and signal intensity changes (Figure 10). However, as shown in Figures 10 and 11, selective line-broadening effects are observed for signals A, D, E, F, and U below Mn^{2+}/tRNA ratios of 0.006 (0.008 mM Mn^{2+}). Smaller line-broadening effects are observed for the other scattered signals. Upon further addition of Mn^{2+}, a general nonselective broadening is found (see top spectra, Figure 10). The phosphate monoester signal A experiences the largest paramagnetic broadening, from 2 Hz at 0 Mn^{2+} to 12.7 Hz at a Mn^{2+}/tRNA ratio of 0.053. The signal is broadened below detection at Mn^{2+}/tRNA of 0.12. Signals D and E broaden from 3 to 5 Hz at low Mn^{2+}

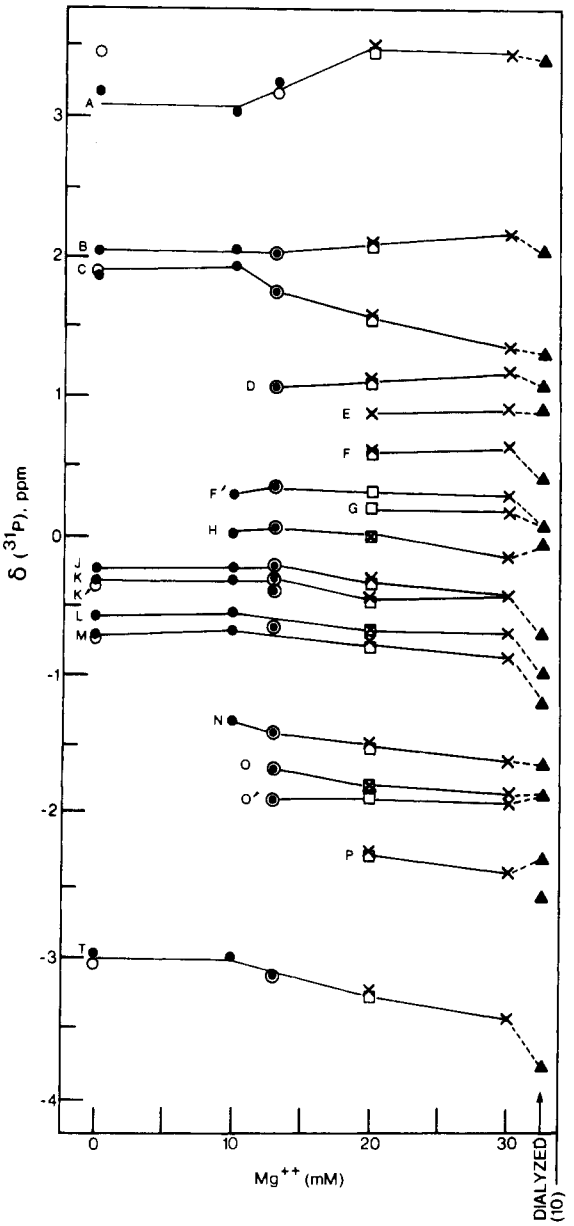

FIGURE 6: Magnesium dependence of the ^{31}P chemical shifts, δ, of the tRNA^{Phe} peaks shown in Figure 4, 39 °C. Sample 1, 145.8 MHz (●); sample 2, 145.8 MHz (○); sample 3, 145.8 MHz (×) and 32.4 MHz (□); sample 3 redialyzed against 10 mM $MgCl_2$, 32.4 MHz (▲).

to 8–10 Hz at Mn^{2+}/tRNA of 0.053. Signal U increases from 6 to 17 Hz with increasing Mn^{2+} concentration. Other signals such as peak B, Figure 10, show little broadening below Mn^{2+}/tRNA ratios of 0.05. (Note that there is some broadening of the unresolved signals in the main cluster upon addition of Mn^{2+}.) Similar line broadening is observed in spectra of tRNA^{Phe} in the absence of Mg^{2+} although poorer signal-to-noise ratios make quantitative analysis difficult. In 13 mM Mg^{2+} samples containing 1 mM EDTA, no line-broadening effects are noted up to Mn^{2+}/tRNA of 0.04.

Discussion

Main Cluster ^{31}P NMR Spectral Assignments. A study of the ^{31}P NMR spectra of tRNA can potentially provide important information on the solution backbone conformation and solution dynamics of these molecules. However, for this NMR probe to be most useful, an assignment of some of the different scattered signals must be made. Such an attempt is made in the following.

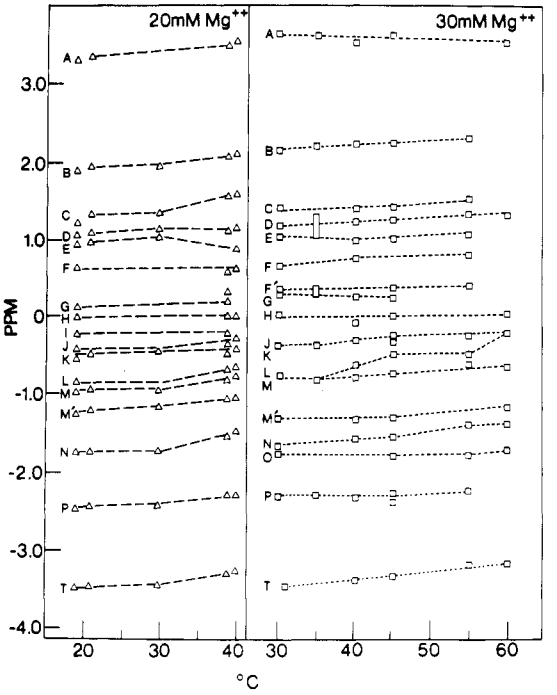

FIGURE 7: Temperature dependence of the ^{31}P chemical shifts, δ, of the peaks identified in Figure 1 for tRNA^{Phe} in standard buffer, 20 or 30 mM $MgCl_2$.

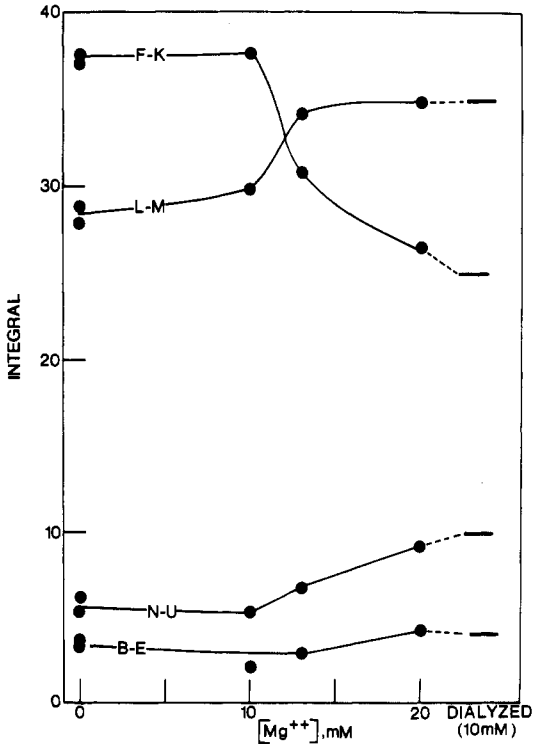

FIGURE 8: Plot of the integrated intensities for various groups of peaks for tRNA^{Phe} in varying $MgCl_2$ buffer, 19 °C.

The yeast phenylalanine acceptor molecule is a class 1 (or D_4V_5) tRNA with four base pairs in the dihyrouridine stem, five bases in the variable loop, and a total of 76 nucleotides. ^{1}H NMR studies (Kearns, 1976; Reid & Hurd, 1979) confirm that in most tRNAs there are about 20 base pairs stabilizing the secondary structure and 7 ± 1 tertiary structure base pairs.

As shown by the ^{31}P spectra in Figure 1, most of the phosphate signal intensity is found in the main-cluster spectral region (0 to −1.5 ppm) rather than in the scattered signals. From model ^{31}P NMR studies on single- and double-stranded

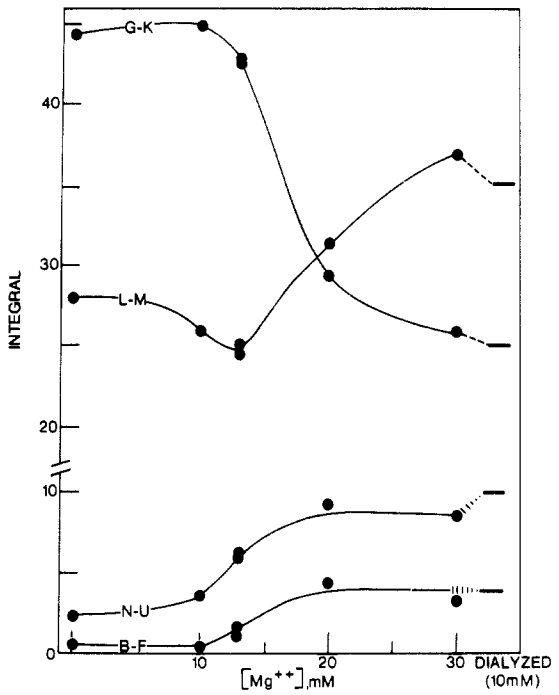

FIGURE 9: Plot of the integrated intensities for various groups of peaks for $tRNA^{Phe}$ in varying $MgCl_2$ buffer, 39 °C.

polynucleotides (Gorenstein et al., 1976a; Gorenstein, 1978, 19781; D. G. Gorenstein et al., unpublished results) and CNDO/2 SCF molecular orbital calculations (Gorenstein & Kar, 1975), the ^{31}P chemical shifts of phosphate diesters in a gauche,gauche (g,g)[2] conformation are 1.4–2 ppm upfield of ^{31}P signals for phosphates in more open (such as gauche,trans) conformations. The helical conformation commonly is $^{-}g,^{-}g$, and as suggested earlier (Gueron & Shulman, 1975; Gorenstein & Kar, 1975; Gorenstein & Luxon, 1979) the main upfield signals L and M at −1 and −1.2 ppm in the $tRNA^{Phe}$ spectrum are likely associated with phosphates having this conformation. Consistent with this interpretation, in the main cluster peaks L and M between −0.9 and −1.5 ppm integrate for 35 phosphates while the X-ray structure indicates that there should be 32 helical $^{-}g,^{-}g$ phosphates that would have ^{31}P signals falling within this chemical shift range. Note that most but not all of these phosphates correspond to the 40 phosphates in the double-helical stems of the cloverleaf model (Figure 12). Surprisingly, in the refined X-ray structure, a number of these double helical phosphates are apparently in non($^{-}g,^{-}g$) conformations (Sussman et al., 1978; Quigley et al., 1975; Stout et al., 1978; Jack et al., 1976).

Peaks J and K between −0.5 and −0.9 ppm have been assigned to unstrained diesters in non-double-helical conformations found largely in the hairpin loops (Gorenstein & Luxon, 1979). These signals integrate for ~16 phosphates, and from the X-ray structure and the calculated correlation of ^{31}P shifts and P–O ester torsion angles, we predict that there should be 14.

Mg^{2+} Dependence to $tRNA^{Phe}$ ^{31}P Spectra. Magnesium stabilizes the functional, native conformation of tRNAs (Fresco et al., 1966) through stabilization of the loops and sharp turns in the tertiary structure (Kim, 1979). While the secondary and tertiary structure of $tRNA^{Phe}$ simultaneously melts with T_m ~65–70 °C in the presence of 10 mM Mg^{2+} (Sprinzl et al., 1974; Bolton & Kearns, 1977; Crothers et al., 1974; Davanloo-Malherbe et al., 1978; Kan et al., 1977; Robillard et al., 1977), sequential melting of structure is observed under low Mg^{2+} and high NaCl conditions. Romer et al. (1969), Riesner et al. (1973), and Coutts et al. (1975) identified five different transitions in the thermal denaturation of $tRNA^{Phe}$ in 30 mM Na^+ without added Mg^{2+} at pH 6.8, conditions comparable to our own. Transition 1 with T_m ~25 °C corresponds to melting of the tertiary structure (leaving the cloverleaf structure largley intact) (Romer et al., 1969). Transitions 2 and 3 with T_m ~35–40 °C (Coutts et al., 1975) correspond to melting of the remaining tertiary structure and the acceptor and anticodon stem, hairpin loop structure. A fourth transition with T_m 45–50 °C corresponds to melting

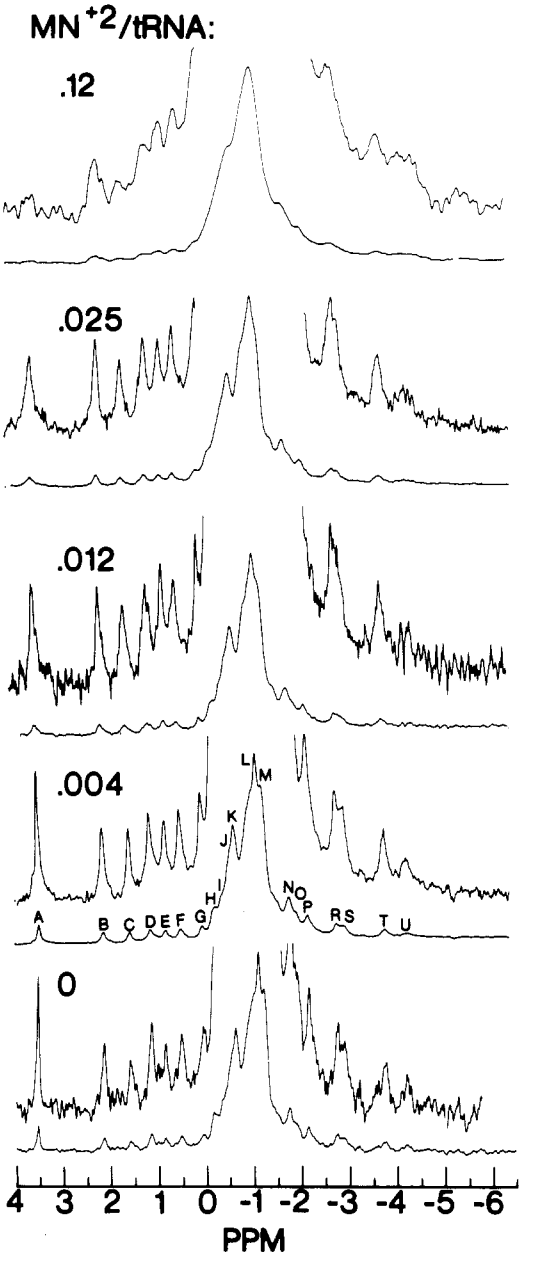

FIGURE 10: ^{31}P NMR spectra of $tRNA^{Phe}$ in 100 mM NaCl, 10 mM cacodylate, 10 mM $MgCl_2$, no EDTA, pH 7.0, 32 °C, and various $MnCl_2$ concentrations (ratio of Mn to tRNA concentration is shown). Number of acquisitions 30 000–40 000. Top spectrum, 2-Hz exponential multiplication line broadening; all others have 0.5-Hz line broadening.

[2] The conformation of a diester is determined by the two dihedral angles, ω and ω', defined by rotation about the phosphate ester bonds, RO–POR'. For $\omega = \omega' = 0°$, the phosphate is in a cis, eclipsed conformation. For a dinucleotide monophosphate, ω' is the conformation about the 3'-O–P bond and ω is the conformation about the 5'-O–P bond. The angles are defined by a counterclockwise rotation (Sundaralingam, 1969). Note we make no distinction between the g,g and $^{-}g,^{-}g$ conformations since they are predicted to have similar ^{31}P chemical shifts.

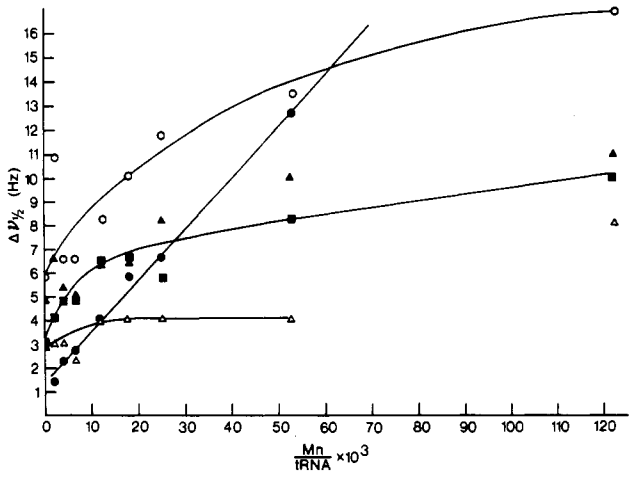

FIGURE 11: Plot of the corrected line width, $\delta\nu_{1/2}$, for various scattered peaks shown in Figure 10 vs. manganese(II) ion concentration in 100 mM NaCl, 10 mM cacodylate, 10 mM $MgCl_2$, pH 7.0, 32 °C. Peak A (●), B (△), D (■), E (▲), U (○).

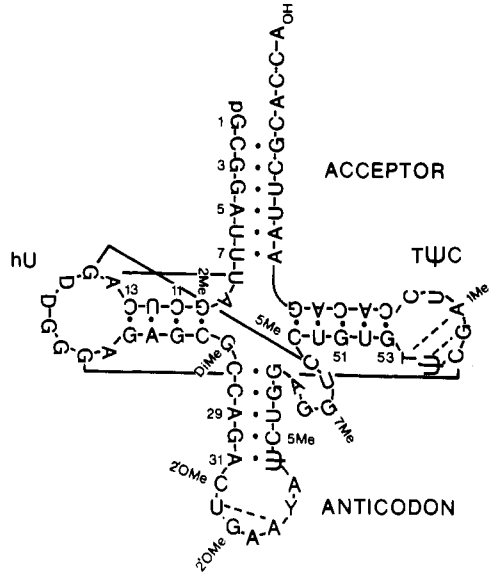

FIGURE 12: Cloverleaf model for yeast tRNA^{Phe}.

of the TψC loop, and a fifth transition with T_m 60–63 °C corresponds to melting of the remaining secondary structure (D stem). No scattered peaks were observed at temperatures greater than 50 °C in the absence of Mg^{2+} (Gorenstein & Luxon, 1979) where significant secondary structure still remains. It is concluded, therefore, that the scattered signals arise from the tertiary interactions.

According to the ^{31}P melting studies, at 19 °C, without added Mg^{2+} both partially denaturated (cloverleaf) and native (tertiary) structures should be present. All of the scattered signals (B–E; N–U) integrate for less than one phosphate each, even at 19 °C (no Mg^{2+}). If the integrated intensity of the scattered signals (B–E; N–U) is utilized as an indicator of tertiary structure, then 64% of the molecules exist in the native conformation at 19 °C (integrated total of 9 phosphates are observed while 14 are expected in the fully native structure; Figure 8). Extra intensity (12–13 phosphates) is observed in the downfield portion of the main cluster (F–K), where signals for the unstrained hairpin-loop phosphates in the cloverleaf structure are expected to be found. About 6 of the double-helix phosphate signals normally observed in the native structures (35 total) are also missing in the 19 °C no magnesium spectrum. Some melting of the stems (fraying at the ends of the double helices) is thus also indicated. At 39 °C, no Mg^{2+}, only 21% of the native structures remains (total of three scattered peaks; Figure 9). Again, the additional intensity (21 phosphates) is found in the region (peaks J/K) which is associated with the hairpin-loop structures of the cloverleaf.

Little change in the 19 °C spectra is observed until ≥10 mM Mg^{2+} has been added. Both the chemical shifts (Figure 5) and integrated intensities of the signals (Figure 8) are unperturbed in 0–10 mM Mg^{2+}. Further addition of Mg^{2+} (to 20 mM) produces a ^{31}P spectrum that looks quite similar to the spectrum of a sample dialyzed against 10 mM Mg^{2+}. Thus the integrated intensity of the scattered signals B–E; N–U in the 20 mM sample is 13 (vs. 14 in the 10 mM dialyzed sample spectrum), and 35 phosphates in the double-helical signals L/M are found under both conditions.

The Mg^{2+} dependence to the ^{31}P chemical shifts at 19 °C (Figure 5) is consistent with the requirement for 10–15 mM Mg^{2+} to stabilize the native structure. With the exception of peak T, the ^{31}P chemical shifts of the other scattered signals (particularly C, E, and P) in 10–15 mM added Mg^{2+} corresponds closely to the ^{31}P shifts of a sample dialyzed against 10 mM Mg^{2+}. Further addition of Mg^{2+} shifts the scattered signals further downfield (A and E) or further upfield (C, F, I, P, and T). The chemical shifts of the downfield main cluster signals (J/K) are little affected by added Mg^{2+} yet even in 20 mM Mg^{2+} are ~0.3 ppm downfield from the signal of a 10 mM dialyzed sample. The double-helical signals L, M, and M′ require 20 mM Mg^{2+} to produce the same upfield shift as found in a 10 mM dialyzed sample.

The requirement for at least 10 mM added Mg^{2+} to produce significant spectral changes in tRNA^{Phe} is consistent with other studies (Cohn et al., 1969; Sander & Ts'o, 1971; Schreier & Schimmel, 1974) that show Mg^{2+} binding to the nonnative structure occurs cooperatively. Apparently at least 4 Mg^{2+}/tRNA is required to stabilize the tertiary structure. In our samples with a concentration for tRNA^{Phe} of 1.4 mM, a minimum of 5.6 mM Mg^{2+} is required before a significant increase in the population of the native molecules occurs. The comparability of the ^{31}P spectra of the 10 mM Mg^{2+} dialyzed sample and the 15 mM added Mg^{2+} sample is consistent with the tight binding of the 4 Mg^{2+} (in the 1.4 mM tRNA sample dialyzed against 10 mM Mg^{2+}, the total Mg^{2+} concentration is ~15 mM: 10 mM free plus 5 mM tightly bound).

Similarly, at 39 °C, at least 10 mM added Mg^{2+} is required to produce any major perturbation in the integrated intensities (Figure 9) and chemical shifts of the tRNA^{Phe} ^{31}P spectra. Increasing Mg^{2+} from 0 to 10 mM decreases the double-helical phosphates (signals L/M) by three and increases the total intensity of the scattered upfield signals (N–U) by a comparable amount. Possibly three helical stem phosphates in the cloverleaf structure become involved in tertiary interactions in 10 mM Mg^{2+}.

At 39 °C the 30 mM Mg^{2+} and 10 mM dialyzed Mg^{2+} samples both have ^{31}P shifts for peak C which are 0.5 ppm upfield from the no Mg^{2+} sample. Otherwise the magnesium dependence to the chemical shifts at 39 °C is similar to that observed at 19 °C.

Gueron & Shulman (1975) had previously concluded that only peak C is sensitive to Mg^{2+}. The discrepency between Gueron and Shulman's work and the present is presumably due to our additional studies at 39 °C and higher Mg^{2+} concentration. Also, as shown in Figure 7, in 20 and 30 mM Mg^{2+} the temperature dependence of the ^{31}P shifts of these signals is essentially eliminated. Thus peak C shifts 0.8 ppm upfield between 20 and 55 °C in the 10 mM Mg^{2+} dialyzed sample. In the 20 and 30 mM samples, this signal shifts slightly

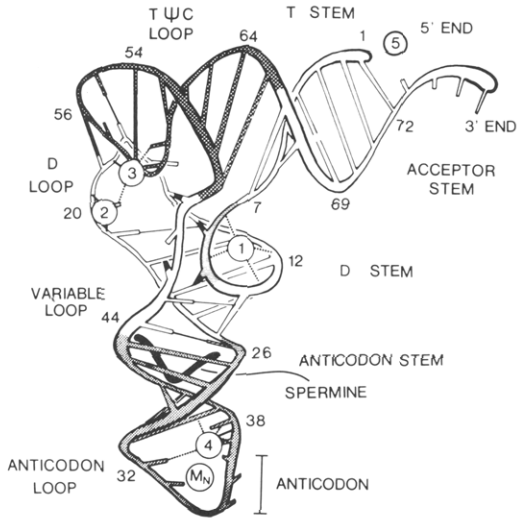

FIGURE 13: Schematic model for three-dimensional structure of yeast $tRNA^{Phe}$ showing sugar–phosphate backbone, base pairs, five magnesium binding sites (numbered circles), manganese site, and spermine binding site (dark curved shape in AC stem). Partially derived from Holbrook et al. (1977) and Kim (1979).

downfield with increasing temperature. Similarly, the 0.4-ppm upfield shift of peaks P and F in the 10 mM Mg^{2+} dialyzed sample in this temperature range disappear in the 20 and 30 mM samples. These three scattered signals and signals A and T are also the most sensitive to magnesium concentration. The phosphate monoester signal A shifts 0.4 ppm downfield at 39 °C with increasing Mg^{2+}. This perturbation of the monoester ^{31}P shift likely reflects a change in the pK of this phosphate in the presence of Mg^{2+}. The Mg^{2+} dependence to the chemical shifts of the scattered diester signals, C, F, P, and T, is likely due to the specific interaction of these phosphates with the tightly bound Mg^{2+} ions.

The X-ray studies on the monoclinic crystals of yeast $tRNA^{Phe}$ (Hingerty et al., 1978; Jack et al., 1977) and the orthorombic crystals (Holbrook et al., 1977; Quigley et al., 1978) have located the four strong magnesium binding sites. The different studies agree that in one site the hexahydrated Mg^{2+} is coordinated to the phosphates of U8, A9, C11, and U12 in the loop between the acceptor and D stems. Coordination is through bridging water oxygens with hydrogen bonding to the phosphates (site 1, Figure 13). A second magnesium binds directly to the phosphate oxygens of A21 and G20 in the D loop. Hingerty et al. (1978) and Holbrook et al. (1977) suggest a third strongly bound magnesium binds directly to the phosphate of G19 and via an intervening water molecule to the bases G20, U59, and C60 (site 3, Figure 13). Quigley et al. (1978) find that this magnesium only binds indirectly to these bases. The fourth magnesium binds directly to the phosphate oxygen of Y_{37} and indirectly to the bases C_{32}, Y_{37}, A_{38} and ψ_{39} (site 4, Figure 13; Quigley et al., 1978; Holbrook et al., 1977), although Higerty et al. (1978) suggest direct binding to the phosphate of G57 in the TψC loop.

As shown in Figure 13, magnesium in sites 2 and 3 cross-linked the D and TψC loops and are likely responsible for the cooperative stabilization of the tertiary structure by magnesium. The temperature dependence to peaks C, F, and P in the 10 mM dialyzed sample likely reflects dissociation (or increased rate of dissociation) at higher temperature for one or more of these four tightly bound, tertiary structure stabilizing magnesium ions. Loss of Mg^{2+} results in a partially denaturated conformation that must retain most of the tertiary structure since UV and ^1H NMR melting studies under these conditions show no change in tertiary structure. Increasing Mg^{2+} to 20–30 mM returns the tRNA to its native state (4 Mg^{2+} bound) even at elevated temperature. Chemical exchange line-broadening effects for peaks T and U had previously provided evidence (Gorenstein & Luxon, 1979) for this premelting conformational transition.

Mn^{2+} Dependence of $tRNA^{Phe}$ ^{31}P NMR Spectra. Selective line broadening is observed for signals A, D, E, and U at low Mn^{2+}/tRNA ratios (<0.010; 0.013 mM Mn^{2+}). Of these signals, only the monoester phosphate signal A is also Mg^{2+} dependent, and certainly the dianionic phosphate is a likely candidate for binding divalent metal ions (site 5, Figure 13). It is surprising that peaks C, P, and T which are Mg^{2+} sensitive and likely reflect the specific tight divalent metal binding sites are not especially Mn^{2+} sensitive.

Proton NMR studies on yeast $tRNA^{Phe}$ of Hurd et al. (1979) have indicated that the first manganese(II) binding site is the phosphate of U8 and A9 (also Mg^{2+} site 1, Figure 13). Chao & Kearns (1977) have also concluded in ^1H NMR studies that the phosphate of U_{33} is the next affected by Mn^{2+} binding, followed by the A_{58}-T_{54} base pair. The X-ray study of Jack et al. (1977) shows that Mn^{2+} can displace magnesium at site 3 but not at sites 1 and 2.

Assignments. Partial assignment of the ^{31}P NMR signals can now be attempted based upon the above metal binding experiments and the modification studies of Salemink et al. (1979). As shown in Table I and discussed earlier, peaks A, C, F, P, and T are all sensitive to magnesium concentration (particularly with regard to their temperature-dependent behavior). It is likely that the phosphates which are Mg^{2+} sensitive are associated with the four tight magnesium binding sites. It is possible, of course, that magnesium binding produces a conformational change and ^{31}P perturbation that is transmitted to a phosphate some distance from the actual Mg^{2+} binding site. Without additional information to the contrary, however, we will take the former, simpler position.

Similarly, we will assume that the phosphates of peaks A, D, E, and U are associated with the tight manganese binding sites identified by ^1H NMR solution studies (Figure 13). The observation of significant broadening for a number of phosphate signals at less than 0.1 Mn^{2+} per tRNA indicates that exchange between the manganese ion and the various metal ion binding sites is rapid on the NMR time scale [as also indicated by earlier Mn^{2+}/$tRNA^{Phe}$ ^1H NMR studies of Hurd et al. (1979) and Chao & Kearns (1977)]. We are adding Mn^{2+} to a high Mg^{2+} solution (Mn^{2+}/Mg^{2+} <1/1000) so that it is doubtful whether we are inducing any conformational change. The broadening of a given phosphate signal by Mn^{2+} will be dependent upon the phosphorus–manganese distance and the residence time of the ion at the binding site. The greatest paramagnetic broadening will thus occur at tight binding sites with direct phosphate oxygen–manganese association. Cho & Kearns (1977) and Hurd et al. (1979) have previously shown that specific ^1H NMR broadening effects are observed at Mn/tRNA ratios less than 0.005. At higher ratios, many other signals (both ^1H and ^{31}P) are broadened.

We know from earlier studies (Gueron & Shulman, 1975; Gorenstein & Luxon, 1979; Salemink et al., 1979) that peaks B–H and N–U are associated with the tertiary structure and peak A is the terminal 5′-phosphate monoester. Salemink et al. (1979) have shown that peak F is lost upon removal of the Y base in the anticodon (AC) loop. Similarly, bovine pancreatic RNase A treatment of $tRNA^{Phe}$ cleaves the ACCA terminus and the phosphodiester bond at U_{33} in the AC loop. As shown in Table I, peaks C, F, and U are lost in this partial hydrolysis of the native structure. In addition, RNase T_1

Table I: Effect of Metal Ion and Modifications on ^{31}P Spectrum of Yeast tRNA^{Phe} and Tentative Identification of Signals

peak[a]	tertiary[b]	Mg^{2+}[c]	Mn^{2+}[d]	−Y[e]	RNase A[f]	RNase T_1[g]	location	tentative identification
A		+	+				AA arm	5′-terminal phosphate
B	+					retained		
C	+	+			lost	retained	AC loop, site 4	Y_{37}
D	+		+			retained		
E	+		+			retained	AC loop, Mn site	U_{33} (or *U*)
F	+	+		lost	lost		AC/D arm, site 1	U_8, A_9, C_{11}, or U_{12}
G	+							
H	+							
I								
J								
K								
L								
M								
N	+					} retained		
O	+					} some		
P	+	+					TψC or D arm, site 2/3	G_{19}, G_{20}, or A_{21}
Q	+							
R	+							
S	+							
T	+	+		} lost			TψC or D arm, site 2/3	G_{19}, G_{20}, or A_{21}
U	+		+	} one	lost	retained	AC loop, Mn site	U_{33} (or E)

[a] See Figure 1 for peak identification. [b] Peaks associated with tertiary structure. [c] Peaks affected by Mg^{2+} ions. [d] Peaks affected by Mn^{2+} ions. [e] Peak lost upon removal of Y base (Salemink et al., 1979). [f] Peaks lost upon partial hydrolysis by RNase A (Salemink et al., 1979). [g] Peaks still remaining after partial hydrolysis by RNase T_1 (Salemink et al., 1979).

treatment of tRNA^{Phe} apparently cleaves the D and TψC loops but does not affect the AC loop. Salemink et al. (1979) have indicated that the only scattered signals remaining after RNase T_1 treatment are B, C, D, E, U, and some in the N–P region.

As shown in Table I, we can conclude from the experiments listed in Table I that peak C is probably in the AC loop (from the RNase A and T_1 data) and is likely one of the Mg^{2+} but not one of the Mn^{2+} binding sites. This description fits best with the phosphate of Y_{37} which is the only Mg^{2+} binding site in the AC. It is also not a Mn^{2+} binding site.

Peak E or U may be tentatively identified as the phosphate of U_{33} since they both are in the AC loop and are not one of the Mg^{2+} binding site but are likely associated with the only Mn^{2+} binding site (U_{33}) in the AC loop. Salemink et al. (1979) have argued that peak U (labeled J_2 in their paper) is likely the hydrogen-bonded phosphate P_{36}. However, as we have previously shown (Gorenstein et al., 1976b; Gorenstein, 1978, 1981), hydrogen-bonding interactions to phosphates produce only small perturbations (<0.5 ppm) and likely are not responsible for the large upfield shift (>3.0 ppm) of peak U. Stereoelectronic effects resulting from tertiary folding of the polynucleotide backbone, however, can readily explain these large shifts (Gorenstein, 1978, 1981).

Peak F is magnesium sensitive and is lost upon RNase A and T_1 treatment. It could thus be associated with the magnesium binding site 1 which is proximate to both the AC and D arms.

Peak U (and T) is unique in showing evidence for chemical exchange line broadening at temperatures below T_m (Gorenstein & Luxon, 1979). Thus the peaks are narrow at low temperature, broaden at intermediate temperature (due to an intermediate rate of chemical exchange), and then narrow again at higher temperature. Salemink et al. (1979) note that peak U is sensitive to sample treatment (phenol extraction), and thus it may reflect the binding of polyamines such as spermine. As shown in Figure 13, X-ray studies have identified a tight spermine binding site in the AC stem (Holbrook et al., 1978; Quigley et al., 1978). The multiple, positively charged base stabilizes the tight turn in the tertiary structure of this loop. Presumably Mg^{2+} ions can substitute in this stabilization in the absence of spermine (assuming our extensive dialysis treatment has removed it). The conformational change monitored by the line broadening and chemical shift changes for peak U are likely associated with a partial unwinding of the AC stem as the positively charged ions are dissociated at higher temperatures (but still below T_m).

Similarly, peaks P and T might be assigned to phosphates in the hinge region of the molecule where the TψC and D loops fold together and where two of the bound Mg^{2+}'s are found (Figure 13, sites 2 and 3). Direct interaction of the phosphates of G_{19}, G_{20}, and A_{21} with the magnesiums are likely responsible for the sensitivity of these peaks to Mg^{2+}. These phosphates are not affected by Mn^{2+} (at low concentrations), and it is believed that these Mg^{2+} binding sites are not one of the three tight Mn^{2+} binding sites. The last magnesium binding site (U_8, U_9) is probably not associated with any of the signals in Table I since the only interaction of the metal ion with the phosphate is through an intervening hydrogen-bonded water. Large bond angle distortions (and ^{31}P shifts) probably require direct coordination of the phosphate with the metal ion.

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