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Biostructural Chemistry of Magnesium Ion: Characterization of the Weak Binding Sites on tRNA^{Phe}(yeast). Implications for Conformational Change and Activity†

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ABSTRACT: The thermodynamics and kinetics of magnesium binding to tRNA^{Phe}(yeast) have been studied directly by ^{25}Mg NMR. In 0.17 M Na^+(aq), tRNA^{Phe} exists in its native conformation and the number of strong binding sites ($K_a \geq 10^4$) was estimated to be 3–4 by titration experiments, in agreement with X-ray structural data for crystalline tRNA^{Phe} (Jack et al., 1977). The set of weakly bound ions were in slow exchange and ^{25}Mg NMR resonances were in the near-extreme-narrowing limit. The line shapes of the exchange-broadened magnesium resonance were indistinguishable from Lorentzian form. The number of weak magnesium binding sites was determined to be 50 ± 8 in the native conformation and a total line-shape analysis of the exchange-broadened $^{25}Mg^{2+}$ NMR resonance gave an association constant K_a of (2.2 ± 0.2) × 10^2 M^{-1}, a quadrupolar coupling constant (χ_B) of 0.84 MHz, an activation free energy (ΔG^*) of 12.8 ± 0.2 kcal mol^{-1}, and an off-rate (k_{off}) of (2.5 ± 0.4) × 10^3 s^{-1}. In the absence of background Na^+(aq), up to 12 ± 2 magnesium ions bind cooperatively, and 73 ± 10 additional weak binding sites were determined. The binding parameters in the nonnative conformation were K_a = (2.5 ± 0.2) × 10^2 M^{-1}, χ_B = 0.64 MHz, ΔG^* = 13.1 ± 0.2 kcal mol^{-1}, and k_{off} = (1.6 ± 0.4) × 10^3 s^{-1}. In comparison to Mg^{2+} binding to proteins (χ_B typically ca. 1.1–1.6 MHz) the lower χ_B values suggest a higher degree of symmetry for the ligand environment of Mg^{2+} bound to tRNA. A small number of specific weakly bound Mg^{2+} appear to be important for the change from a nonnative to a native conformation. Implications for interactions with the ribosome are discussed.

Although the structural and catalytic roles of transition-metal centers in biological molecules have been the subject of much attention (Singer & Ondarza, 1978), similar detail is lacking in our knowledge of the biochemistry of the most abundant metal ions in biology (Forsen & Lindman, 1978), the alkali and alkaline-earth ions, Na^+, K^+, Mg^{2+}, and Ca^{2+}. Williams (1970) has summarized the area in a recent review. In large part this can be attributed to the absence of convenient physical and spectroscopic properties (electrochemistry, ESR, electronic absorbtion, luminescence) to probe their immediate environment in solution. The application of NMR methods to the study of sodium and potassium metabolism, in particular, has provided greater insight on the chemistry of these ions in living systems [e.g., James and Noggle (1969) and Bleam et al. (1980)]. Magnesium and calcium, however, serve distinct biological roles from the alkali metals and are less readily studied by NMR. Excellent reviews outlining the application of $^{44}Ca^{2+}$ and $^{25}Mg^{2+}$ NMR to monitor ion binding to proteins and smaller ligands are available (Drakenberg et al., 1983; Forsen et al., 1981; Vogel & Forsen, 1986; Drakenberg et al., 1984; Forsen & Lindman, 1981; Neurohr et al., 1983).

Magnesium is vital to the regulation of structure in tRNA and rRNA (Fresco et al., 1966; Lake, 1985) and is an essential cofactor for many RNA and DNA processing enzymes (Linn

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& Roberts, 1985) and for those enzymes using ATP, ADP, or AMP as a substrate. Examples have been identified where Mg^{2+} is believed to complex with and activate the enzyme prior to phosphoryl transfer (e.g., ATPase in active transport) (Ochiai, 1987) and others where the ion remains bound to the nucleotide fragment and simply catalyzes the transfer of a phosphate unit. In many cases Mg^{2+} would appear to be uniquely qualified as an activator of enzyme activity. Magnesium binding to tRNA has been studied by ^1H NMR (Kearns, 1976; Li et al., 1987), fluorescence techniques (White & Cantor, 1971; Lynch & Schimmel, 1974; Wolfson & Kearns, 1974), and equilibrium dialysis (Stein & Crothers, 1976a,b; Danchin, 1972; Schreier & Schimmel, 1974; Yang & Crothers, 1972). It is recognized that Mg^{2+} binds to the phosphodiester backbone rather than to the individual bases although this is not necessarily true for other transition or alkaline-earth metals (Saenger, 1984). From previous work on tRNA two types of binding modes [strong ($K_a \sim 10^5$ M^{-1}) and weak ($K_a \sim 10^2$ M^{-1})] have been identified (Stein & Crothers, 1976a,b; Danchin, 1972; Schreier & Schimmel, 1974). Detailed crystallographic studies on $tRNA^{Phe}$(yeast) have revealed the location of strongly bound Mg^{2+} and the binding sites for other transition- and heavy-metal ions (Jack et al., 1976, 1977). Little information has been gathered on the weaker binding sites. There is poor agreement among studies of the strong binding sites with regard to the determination of the number of bound ions, the association constants, and the influence of cooperativity (White & Cantor, 1971; Lynch & Schimmel, 1974; Wolfson & Kearns, 1974; Manning, 1979; Stein & Crothers, 1976a,b; Danchin, 1972; Schreier & Schimmel, 1974; Yang & Crothers, 1972; Saenger, 1984; Stein & Stein, 1976; Horrocks & Albin, 1983), which can be attributed to the use of different solvent conditions (low salt or high salt) or distinct metal ions as analogues. Previous evidence for cooperativity in the binding of Mg^{2+} to certain tRNAs is likely to be a consequence of induced conformational folding in the absence of background salt (Stein & Stein, 1976). In >0.1 M NaCl the native conformation is adopted and no cooperativity is evident. The strong binding sites on $tRNA^{Phe}$ have been extensively characterized in the solid state by crystallography; however, the weak binding sites have not received detailed attention, including the determination of the number of sites and the dependence of this on conformation, the structural nature of these sites, and the kinetics and thermodynamics of binding. In this paper we discuss the coordination chemistry of the weak magnesium binding sites on $tRNA^{Phe}$(yeast) in both native and nonnative conformations and also directly address questions of cooperativity in binding and discuss the potential role of these sites as triggers of allosteric change and ribosome function.

The application of ^{25}Mg NMR as a direct probe of ion binding to proteins and small molecules has been developed extensively by Forsen and co-workers (Drakenberg et al., 1983; Forsen et al., 1981; Tsai et al., 1987; Vogel & Forsen, 1986; Drakenberg et al., 1984). An important early application in studies of magnesium binding to small complexes, DNA, and proteins was carried out by Bryant and co-workers (Bryant, 1972; Rose et al., 1980; Bleam et al., 1980). The suggestion by Bryant that magnesium NMR might be used in the study of magnesium binding proteins has been borne out by recent work. Although studies of this type are still limited, the potential utility of the technique for the determination of association constants (K_a), exchange rates ($k_{on/off}$), and activation parameters (ΔG^*, ΔH^*) has been demonstrated. The inherent errors in such determinations are not insignificant (±10–20%); however, there are few alternative methods for the determination of such data in the study of electronically and magnetically silent metal ions. Stein and co-workers (Stein & Crothers, 1976a,b; Stein & Stein, 1976) have used equilibrium dialysis to determine the binding constants to both $tRNA^{Glu_2}$ and $tRNA^{fMet}$ from *E. coli* and obtained evidence for one strongly bound Mg^{2+} ($K_1 = 7.5 \times 10^4$ M^{-1} and 3.0×10^4 M^{-1}, respectively) and several weaker sites ($K_2 = 8.3 \times 10^2$ M^{-1} and 4×10^2 M^{-1}, respectively). ^{25}Mg NMR ($I = 5/2$) is of most use for studies of metal ion binding where $K_a \leq 10^4$ M^{-1} (Neurohr et al., 1983) and is therefore ideally suited to monitor the weak binding sites of tRNA.

Materials and Methods

Materials. $tRNA^{Phe}$(yeast) was obtained from Sigma Chemical Co. The samples were essentially free of magnesium ion (<0.2 equiv) as determined by atomic absorption. ^{25}MgO (98 atom % ^{25}Mg) was obtained from Oak Ridge National Laboratory. A stock solution of $^{25}Mg^{2+}$ (~0.1 M) was prepared by dissolving the oxide in 0.1 M $HClO_4$ and the pH was adjusted to 7 with NaOH. pH measurements were made by using an Accumet pH Meter 910 equipped with a Ross combination pH electrode (Orion Research Inc.). Deionized water used in all procedures was obtained by passing distilled water through a Barnstead nanopure system.

^{25}Mg NMR Experiments. ^{25}Mg spectra were recorded at 18.374 MHz on a Bruker MSL 300 spectrometer. Typical spectra were obtained without sample spinning by using the RIDE pulse sequence to reduce the effects of acoustic ringing (Ellis, 1983). Normally two left shifts were applied to the data prior to Fourier transformation. A standard 0.3 M $MgCl_2$ solution gave a signal with a line width of 3.5 Hz, in good agreement with literature precedent (Lindman et al., 1977). The 90° pulse width was 30 μs; the acquisition time was 26 ms for broad lines and 86 ms for narrow lines with spectral widths of 10000 Hz and 3000 Hz, respectively, with a preacquisition delay of 60 μs. The deadtime of the instrument is ca. 20 μs using the acquisition parameters noted above. Since this is much shorter than the relaxation time ($1/R_{2B}$) of the observed signals, we could readily acquire spectra of resonances with line widths of several hundred hertz. A Lorentzian line broadening of 50 or 100 Hz was typically applied to data, depending on the experiment.

Generally both magnesium titration and variable-temperature (VT) experiments were carried out for each sample. In the case of $tRNA^{Phe}$ the experiments were repeated in the presence and absence of background 0.17 M NaCl(aq). Typically the concentration of tRNA was ca. 0.1 mM, determined by measuring the optical density at 260 nm and using the standard absorbance values provided by the supplier (Sigma). Titration experiments were carried out at 298 K, with successive additions from the stock solution of $^{25}Mg^{2+}$. In the later portion of the titration, when a good signal/noise was attainable within a reasonable time period, additions of Mg^{2+} were made from an ~1 M $MgCl_2$ solution (adjusted to pH 7 with NaOH). In all cases the concentrations of stock solutions were accurately determined by atomic absorption. Variable-temperature experiments (from 5 to 50 °C) were performed with a fixed $[Mg^{2+}]/[tRNA]$ ratio. The temperature was not raised above 50 °C in the case of RNA experiments because of the likelihood of denaturation. The values for $\Delta\nu_{1/2}$ obtained both before and after heating were similar.

Theoretical Considerations of Magnesium NMR and Data Analysis. The relative receptivity of ^{25}Mg at natural abun-

dance is higher than that of ^{13}C, and the use of isotopically enriched material significantly improves the sensitivity of the experiment. Although the large quadrupole moment of ^{25}Mg ($I = 5/2$, $Q = 0.22 \times 10^{-24}$ cm^2) makes studies using this nucleus technically demanding in comparison to other biologically relevant ions (e.g., $^{43}Ca^{2+}$, $I = 7/2$, $Q = -4.9 \times 10^{-26}$ cm^2), data analysis by total line-shape analysis of the exchange-broadened signal has been facilitated by the results of recent detailed theoretical work (Neurohr et al., 1983; Halle & Wennerstrom, 1987). The chemical shift range of ^{25}Mg is small (Halle & Wennerstrom, 1981) and data are obtained by a total line-shape analysis of the quadrupole relaxed resonance. For relatively small RNAs such as tRNA we estimate that the data should lie close to the near extreme narrowing limit ($\tau_c\omega_0 \leq 1.5$). The explicit calculation is described below and is supported by our experimental results.

The line-shape analysis programs that we use were developed according to the theoretical interpretation of Halle and Wennerstrom (1981) by Dr. T. Drakenberg (University of Lund, Sweden) and are capable of analyzing data in this exchange regime. This treatment utilizes the Bloch equations to derive a band-shape expression for an exchange system. Several parameters are required to fully define the line shape of a resonance. These include the chemical shifts of free and bound ions (for Mg^{2+} the chemical shift is normally similar for free and bound forms). The populations of free and bound ions are dependent on [Mg^{2+}], [RNA], and the binding constant K_a, and so these parameters are introduced into the analysis routine. The transverse relaxation rate for bound magnesium can be described, according to Halle and Wennerstrom (1981), by

$$R_{2B} = \frac{3\pi^2}{10}\chi_B{}^2\frac{2I+3}{I^2(2I-1)}\left(0.3\tau_c + \frac{0.5\tau_c}{1+(\omega\tau_c)^2} + \frac{0.2\tau_c}{1+(2\omega\tau_c)^2}\right) \quad (1)$$

where χ_B is the quadrupolar coupling constant for bound $^{25}Mg^{2+}$ and τ_c is the correlation time for bound magnesium. In the absence of direct evidence it is often assumed that τ_c for the metal ion is equal to the τ_c for the biological macromolecule. The justification for such an assumption derives from the following considerations. In those cases where thermodynamic data have been obtained for magnesium binding to other proteins and polynucleotides there has been good agreement with the values of parameters obtained by ^{25}Mg NMR using the assumed τ_c values (Drakenberg et al., 1983; Forsen et al., 1981; Vogel & Forsen, 1986; Drakenberg et al., 1984; Tsai et al., 1987). Evidence would suggest that the metal ion is normally tethered to the biological macromolecule and is not loosely held in an ill-defined cavity (Drakenberg et al., 1983; Forsen et al., 1981; Vogel & Forsen, 1986; Drakenberg et al., 1984; Tsai et al., 1987; Linse et al., 1987). However, should the ion possess a small amount of internal freedom it can be demonstrated that an uncertainty in τ_c of an order of magnitude does not greatly influence the derived parameters and normally leads to an underestimation of χ_B but does not affect K_a or ΔG^*. Most importantly, with regard to the question of whether the treatment of Halle and Wennerstrom is appropriate, it should be noted that the protein τ_c is an upper limit and $(\omega_0\tau_c)_{free} < (\omega_0\tau_c)_{tRNA}$; therefore, the near-extreme-narrowing conditions are maintained. Of the remaining parameters in eq 1, χ_B is given by e^2Qq_{zz}/h, where Q is the quadrupole moment and q_{zz} is the electric field gradient for a given nucleus. χ_B might therefore be envisioned as a measure of the asymmetry at the metal ion. The temperature dependence of k_{off} is assumed to follow standard transition-state theory:

$$k_{off} = (kT/h)e^{-\Delta G^*/RT} \quad (2)$$

This manifests itself in the line-shape equation through the dependence of line shape on the relative rate of exchange between free and bound forms. An estimate of τ_c can be made from available optical and magnetic resonance data for RNAs. For example, at a field strength of 18.374 MHz, τ_c for $tRNA^{Phe}$ in aqueous solution is ca. 24.5 ns; therefore $\tau_c\omega_0 \sim 2.87$ (Tao et al., 1970; Beardsley et al., 1970) and lies close to the near-extreme-narrowing limit ($\tau_c\omega_0 \leq 1.5$). The error involved with a $\tau_c\omega_0$ slightly greater than 1.5 is not significant (Tsai et al., 1987). The experiment therefore monitors the change in line width of the exchange-broadened resonance of free Mg^{2+}. From consideration of the variable-temperature behavior it is clear that magnesium binding to the weak sites on tRNA is in the slow-exchange regime. Under such limiting conditions, relaxation behavior should be dominated by a single-exponential term and resonances should approximate to Lorentzian behavior. A Lorentzian line shape is not, however, assumed in the analysis program. The data obtained in this study could be readily fit to a Lorentzian line form. The observation by Bryant and Record (Bryant, 1972; Rose et al., 1980; Bleam et al., 1980) of a strict non-Lorentzian line shape for interactions of magnesium with calf-thymus DNA was presumably due to the large size and cylindrical shape of this molecule, giving rise to an adverse correlation time.

Data were analyzed from an iterative least-squares fit of digitized NMR spectra. Zero filling was employed where necessary to better define the peak. Typically, each spectrum was defined by 30 points and the best combination of ΔS^*, ΔH^*, χ_B, and K_a was found by an iterative procedure. Consideration of the variation of line width ($\Delta\nu_{1/2}$) with [Mg^{2+}]/[RNA] yields values for the association constant (K_a) and the quadrupole coupling constant (χ_B), while the activation free energy and components (ΔG^*, ΔH^*, and ΔS^*) and the off-rate (k_{off}) can be derived from the variation of line width with temperature (Tsai et al., 1987). It should be noted that the titration and temperature data must be analyzed cyclically because of the interdependence of K_a and ΔG^*. The analysis routine, considering all data sets, gives rise to unique values for each of the aforementioned parameters. In agreement with other studies (Tsai et al., 1987) we have found that slight deviations from the optimal values of one parameter (≥5%) yield bad fits. Normally a Lorentzian line-broadening function of 50–100 Hz was applied to data prior to Fourier transformation. Since this is only a fraction of the total range of natural line widths, which typically vary from 30 to 800 Hz (see Figure 1a,b), a minimal error is introduced. Also, the natural line shapes are dominated by a single Lorentzian relaxation function as expected in the near-extreme-narrowing limit. The line broadening does not therefore produce artifactual data.

Determination of the Number of Mg^{2+} Bound to tRNA. The number of weak binding sites was estimated by determining the $[Mg^{2+}]_{bound}$ when a large excess of Mg^{2+} was added to a RNA solution of known concentration. A concentration of magnesium $[Mg^{2+}]_{total}$ was used that saturated the binding sites on tRNA and was estimated from the titration data (typically a 250-fold excess was used; Figure 1a,b). The number of bound ions was then determined by filtering the solution through a centricon (Amicon Corp.; 30-kDa cutoff) to remove RNA and bound Mg and determining the $[Mg]_{free}$ by atomic absorption spectroscopy (where $[Mg^{2+}]_{bound} = [Mg^{2+}]_{total} - [Mg^{2+}]_{free}$). The ratio [$Mg^{2+}$]/[RNA] gave the

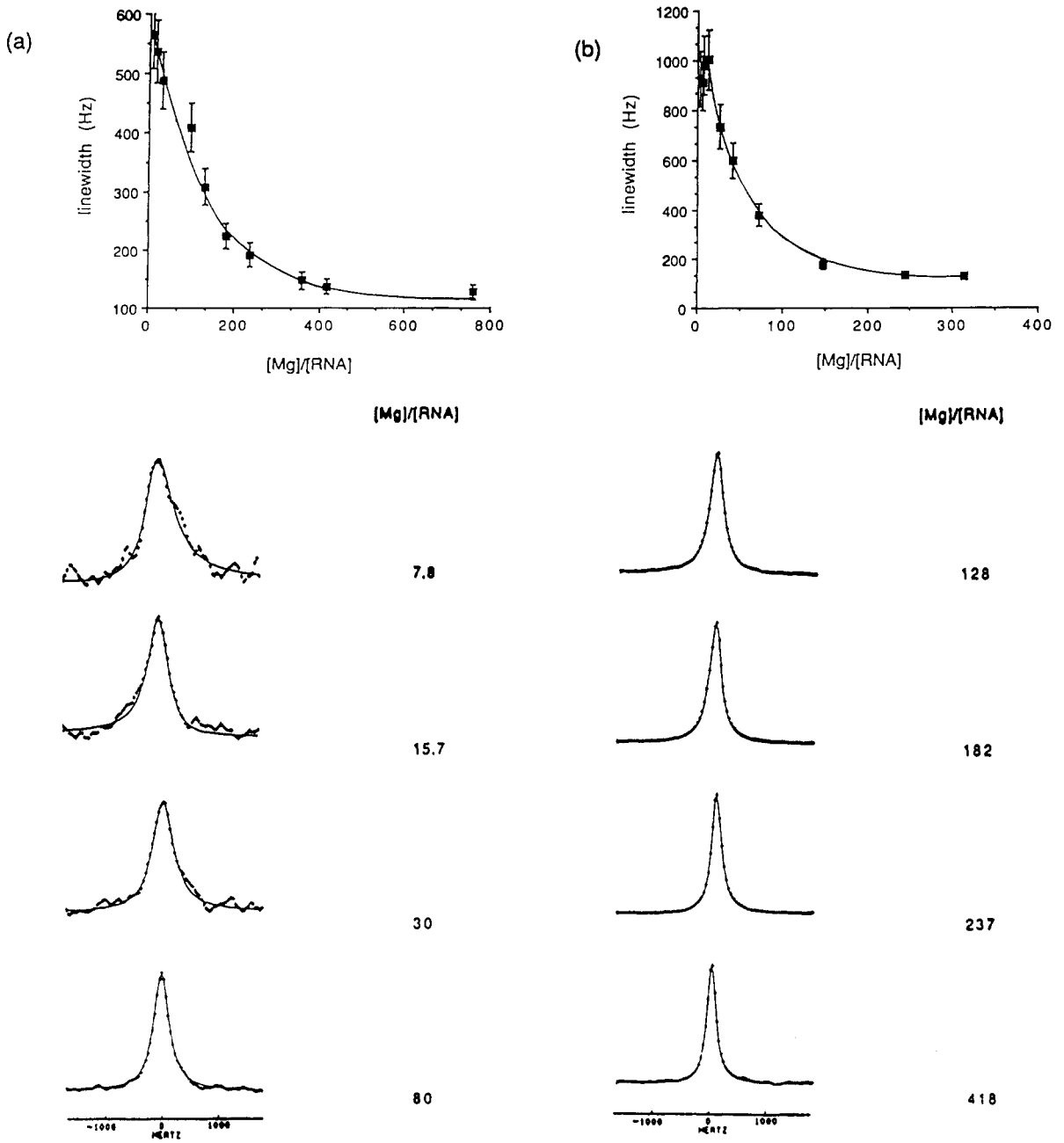

FIGURE 1: Magnesium titration curve obtained at 303 K: (a) in 0.17 M NaCl, $[Mg^{2+}]$ varied from 0.51 to 45.8 mM, [tRNA] varied from 65 to 59 μM; (b) in the absence of background salt, $[Mg^{2+}]$ varied from 0.14 to 44.5 mM, [tRNA] varied from 143 to 139 μM. $\Delta\nu_{1/2}$ includes a line broadening of 100 Hz. Typical spectral parameters were as follows: spectral digitization, SW = 10000 Hz, SI = 512 W, TD = 512 W; pulse width = 90° (30 μs). The experimental points are shown relative to a theoretical curve obtained by joining calculated points from the fitting analysis. A typical series of ^{25}Mg NMR spectra taken at various ratios of $[Mg^{2+}]/[tRNA]$ from the data collected for the plot shown in (a) are illustrated at the bottom of the figure. $\Delta\nu_{1/2}$ included a line broadening of 100 Hz. The trace indicated by (+) is the digitized spectrum with the calculated line shape (---) superimposed.

total number of sites (strong + weak). Since the number of strong sites had been determined above, the number of weak sites was readily estimated with an error of ca. ±10%.

Atomic Absorption. Measurements were made on a Perkin-Elmer atomic absorption spectrometer (AA) using an air/acetylene fuel mixture. The Mg lamp was operated at 285.2 nm, slit width 0.7 nm, and the instrument was calibrated against a series of solutions containing 0.1, 0.3, and 0.5 ppm of Mg^{2+}, prepared by successive dilutions of a 1000 ppm Mg^{2+} standard solution (GFS Chemicals) with nanopure water that contained 1% concentrated HCl. Volummetric glassware was pretreated with an acid wash. Known volumes of the magnesium stock solutions were diluted to give an approximate concentration between 0.15 and 0.5 ppm in Mg^{2+}. (Note: the linear working range of the AA for Mg is below 0.5 ppm.) Both integrated signals and nonintegrated signals were used to determine the concentrations of Mg^{2+} to ±2.5%.

Results

Magnesium Binding to $tRNA^{Phe}$. Titration Experiments (Determination of the Number and K_a's of Weakly Bound Ions). In 0.17 M NaCl solution tRNA adopts its native conformation while nonspecific binding by Mg^{2+} is prevented. It has previously been demonstrated that magnesium binding is noncooperative under these conditions (Stein & Crothers, 1976a,b; Schreier & Schimmel, 1974). Figure 1a shows a typical series of spectra in the digitized form used for data analysis. Although theoretically non-Lorentzian, each plot could be fit well to a Lorentzian line-shape function. The theoretical treatment does not, however, assume Lorentzian

Table I: Thermodynamic and Kinetic Data for Magnesium Binding to Weak Coordination Sites on $tRNA^{Phe}$(yeast) in Native and Nonnative Conformations

conformation	K_a (M^{-1})	ΔH^* (kcal/mol)[a]	ΔG^* (kcal/mol)	χ_B (MHz)	R_{2B} (kHz)[a,b]	$\Delta\nu_{1/2}$ (kHz)[a,b]
native[c]	$(2.2 \pm 0.2) \times 10^2$	2.7	12.8 ± 0.2	0.84 ± 0.1	6.2	2.0
nonnative[d]	$(2.5 \pm 0.2) \times 10^2$	2.9	13.1 ± 0.2	0.64 ± 0.1	7.2	2.3

[a] These values provided the best fits for the association constants (K_a) and quadrupole coupling constants (χ_B). An error of 15–20% is estimated for these parameters. [b] R_{2B} is the relaxation rate of bound magnesium; $\Delta\nu_{1/2}$ is the resonance line width of bound magnesium. [c] In 0.17 M NaCl, pH 7.0, 303 K, assuming the noncooperative binding of 50 magnesium ions. [d] No background salt added, pH 7.0, 303 K. Data were determined from the noncooperative binding region ($[Mg^{2+}]/[RNA] > 12$) for 73 magnesium ions.

line forms. At low concentrations of Mg^{2+} (2 equiv) no signal was detected, even after extended data acquisition. This is indicative of slow exchange at strong binding sites. We estimate below a $\Delta\nu_{1/2}$ of 2 kHz for the bound ion, which is too broad to detect under the experimental conditions employed and with the signal:noise ratio's that might reasonably be determined. After the addition of 4 equiv, however, a weak resonance was detected (Figure 2). From these titration studies we estimate the number of strong binding sites to be 3 ± 1. This is in agreement with the number determined by crystallography (Jack et al., 1976, 1977) but not for the values determined for $tRNA^{Glu_2}$ and $tRNA^{fMet}$ from *E. coli*. Measurements by equilibrium dialysis suggested one strong binding site (Stein & Crothers, 1976a,b; Danchin, 1972) for these RNAs and it was reasoned that binding of sodium ions might block the remaining sites (Stein & Stein, 1976). These authors also noted, however, that different tRNAs might possess intrinsically different metal binding characteristics, and our results confirm this conclusion. The variation of line width at half-height ($\Delta\nu_{1/2}$) has been plotted against $[Mg^{2+}]/[RNA]$ and is shown in Figure 1. Upon increasing $[Mg^{2+}]$ a relatively broad resonance was observed that sharpened systematically with increasing $[Mg^{2+}]$. The decrease in line width is due to magnesium binding to specific weak binding sites on the tRNA. Before fitting the data with the analysis routine described above, we required an estimate of the number of weak sites involved in magnesium binding. This was estimated by determining the $[Mg^{2+}]_{bound}$ when a 250-fold excess of Mg^{2+} was added to an RNA solution of known concentration. The number of bound ions was determined following rapid centricon ultrafiltration (Amicon Corp.; 30-kDa cutoff) to remove RNA and bound Mg^{2+} and determination of the $[Mg]_{free}$ by atomic absorption spectroscopy (where $[Mg^{2+}]_{bound} = [Mg^{2+}]_{total} - [Mg^{2+}]_{free}$). The binding constant was large enough to prohibit significant dissociation of bound Mg^{2+} during filtration and the ratio $[Mg^{2+}]_{bound}/[RNA]$ gave the total number of sites (strong + weak). Since the number of strong sites had been determined above, the number of weak sites could be estimated. The average from three independent experiments was 50 ± 8, in reasonable agreement with the estimated 39 sites from crystallographic data (Jack et al., 1977). A fit to the titration data in Figure 1a gave the following binding parameters for the weaker sites: $K_a \sim (2.2 \pm 0.2) \times 10^2$, $\chi_B = 0.84$ MHz, assuming 50 binding sites and optimizing the data using the activation parameters from the variable-temperature experiments described below. The error in the number of binding sites is in keeping with the overall accuracy of the experiment and the subsequent results and conclusions are not affected.

In the absence of Na^+(aq) tRNA adopts a "nonnative" conformation (Stein & Crothers, 1976a,b; Danchin, 1972; Schreier & Schimmel, 1974). Both τ_c (25.2 ns) and the effective molecular volume (116 000 Å^3) have been determined by fluorescence polarization measurements and are very similar to the values for native tRNA (24.5 ns and 114 000 Å^3, respectively) (Tao et al., 1970; Beardsley et al., 1970). A resonance was observed after the addition of 1 equiv of magnesium, and so the strong binding domains are not as well defined in the nonnative conformation. Presumably those strong magnesium binding sites that serve to stabilize tertiary structure by cross-linking adjacent bases in loop regions and bridging two single-strand regions (Figure 3) are perturbed through electrostatic repulsion between areas of the negatively charged phosphodiester backbone. Cooperative behavior was observed in the initial stages of the titration. The addition of up to 12 equiv of Mg^{2+} leads to an increase in line width. A total of 85 ± 10 magnesium ions were determined by the atomic absorption procedure outlined above, and 73 ± 10 are identified with the weak binding sites. Subsequent additions lead to a sharp decrease in line width, although the calculated association constant per site, K_a $2.5 \pm 0.8 \times 10^2$ M^{-1}, is comparable to that found in the native structure, as is the quadrupolar coupling constant $\chi_B = 0.64$ MHz, due to the increased number of weak binding sites in the absence of background salt. It is emphasized that the association and activation parameters determined above and below are average values for individual sites and are not attributable to binding of Mg^{2+} to tRNA as an overall unit. The question of how unique the binding parameters for each site might be shall be discussed below. The results from variable-temperature (VT) data were used in a cyclic iterative procedure to optimize the results from titration and VT experiments.

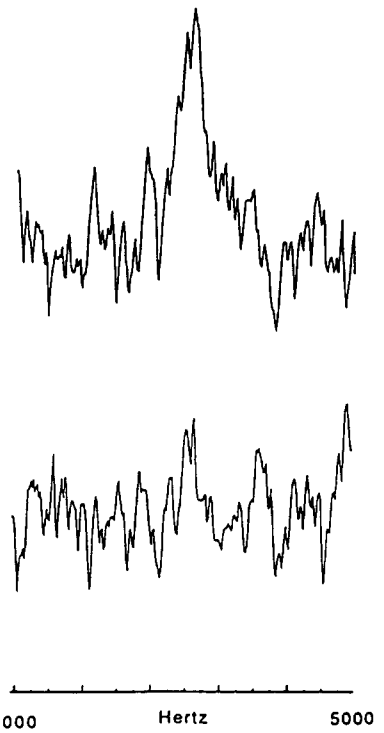

FIGURE 2: Spectra taken after the addition of (a, top) 2.0 and (b, bottom) 3.9 equiv of $^{25}Mg^{2+}$ to a 65 μM solution of tRNA in 0.17 M NaCl. $\Delta\nu_{1/2}$ includes a line broadening of 100 Hz.

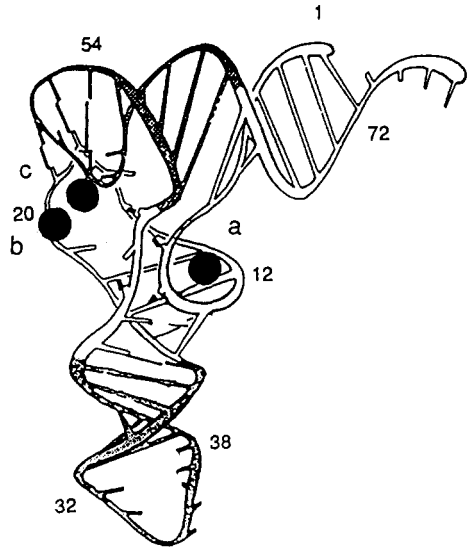

FIGURE 3: Three strong binding sites resulting from binding to backbone phosphates (a) on U8/A9/C11/U12 and (b) G20/A21 and (c) from bridging two single-strand regions by binding to G19 and hydrogen bonding via the solvent hydration shell to oxygens and nitrogens on U59, C60, and G20 (Jack et al., 1977). The weakly bound Mg^{2+} sites are likely to be predominantly located in the grooves of the α-helices. Adapted from Jack et al. (1977) and Quigley et al. (1978).

Magnesium Binding to tRNA^{Phe}: Variable-Temperature Experiments. By use of a procedure similar to that outlined above, the results from variable-temperature experiments were optimized in line with the titration data. A 200-fold excess of Mg^{2+} was used to saturate the binding sites so that the experiment might reflect differences in exchange rates rather than relative populations of bound and free ion. In the native conformation the line width (Hz) demonstrated a slight temperature dependence (Figure 4), showing a slow increase with increasing temperature. The system therefore lies in the slow exchange region with a low activation enthalpy and the best fits to the data gave $\Delta H^* = 2.7 \pm 0.2$ kcal and $\Delta G^* = 12.8 \pm 0.2$ kcal. An off-rate for magnesium binding of $k_{off} = (2.5 \pm 0.4) \times 10^3$ s^{-1} was estimated from the optimized K_a and ΔG^* [$k_{off} = (kT/h) \exp(-\Delta G^*/RT)$]. The spectrum should show two signals: one due to bound ion and another due to an exchange-broadened free magnesium resonance. However, only one Lorentzian peak is observed since the line width of bound magnesium is too broad to detect with the instrumental conditions employed ($\Delta\nu_{1/2}$ = 2 kHz; determined by using eq 1 and $R_{2B} = \pi\Delta\nu_{1/2}$).

In the absence of background salt a slightly more pronounced temperature dependence was observed, presumably due to the larger number of magnesium centers since in every other respect the data are comparable (Figure 4). Magnesium is again in slow exchange and the observed line widths are due to free Mg^{2+}, broadened by exchange. The best fit to the data, optimizing both the titration and variable-temperature data, gave $\Delta H^* = 2.9 \pm 0.2$ kcal, $\Delta G^* = 13.1 \pm 0.2$ kcal, and $k_{off} = (1.6 \pm 0.4) \times 10^3$ s^{-1}. The line width of bound magnesium is calculated to be 2.3 kHz and so is broadened into base-line noise.

A possible source of error might arise from any dependence of K_a on temperature. It has been previously shown from experiments on a number of phosphate-bearing molecules that the variation in K_a over the temperature range employed in these experiments is less than a factor of 2 (Kahn & Martell, 1966, 1967). Additionally, the use of a large excess of Mg^{2+} will offset this effect. Any correction to the activation energies

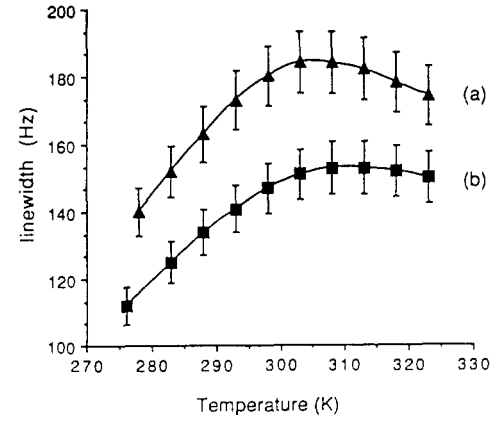

FIGURE 4: Variation of line width with temperature. $[Mg^{2+}] = 12.9 \pm 0.4$ mM, [RNA] = 66 ± 1 μM with (a) no background salt and (b) 0.17 ± 0.02 M NaCl, both at pH 7.0. Other spectral parameters were similar to those detailed in the legend to Figure 1. The FID was zero filled once prior to digitization and $\Delta\nu_{1/2}$ includes a line broadening of 50 Hz.

as a result of this difference is minor and within the quoted error limits. A further source of error might originate from a distribution of exchange rates over the large number of weak binding sites. We have assumed that the large majority of weak binding sites on tRNA are similar with regard to ligand environment, and so the binding parameters should also be similar. In viewing the tertiary structure of tRNA, it is clear that this is basically true; the apparently complex folded structure should not be confused with the basically simple double-stranded RNA from which it is constructed. It follows that a large range of K_a, ΔG^*, and ΔH^* would not be expected for the bound ions and so these parameters are assumed comparable for each site. The very small number of unusual binding sites that exist would not significantly influence the overall results derived from the analysis. In any event the analysis is valid for comparison of native versus nonnative conformations.

DISCUSSION

Previous fluorescence studies have suggested similar conformations for the native and nonnative structures (Tao et al., 1970; Beardsley et al., 1970); however, our data suggest that the ion binding properties are distinct in several ways. In the absence of background counterions (Na^+ and Cl^-) there is an increase in the number of Mg^{2+} centers bound to tRNA. Mg^{2+} binds more strongly to polynucleotides than Na^+ (Bleam et al., 1980), and so the latter would appear to compete effectively with magnesium at higher concentrations under native conditions since the number of magnesium binding sites falls in the presence of excess Na^+. This may be an oversimplification, however, and might be explained in two ways. First, there may indeed exist a subset of very weak labile magnesium binding sites that are populated in the absence of Na^+ but are readily displaced in the presence of excess Na^+. Second, the data are also readily explained by the occurrence of additional magnesium binding sites that are not present in the native conformation, which may be conformationally hidden. In the absence of background salt, the RNA helices might "breath" in order to reduce electrostatic interactions between these chains. Conformational opening of the overlapping helices (Figure 3) will result in additional accessible binding sites in the grooves of the RNA backbone. The choice between these two possibilities will have obvious relevance to our data analysis since the former considers a system with two classes of weak binding site with distinct numbers of ions populating each. The very weak sites would have to possess binding constants $\ll$200,

which in turn suggests a larger value for k_{off} (a lower k_{on} is not compatible with a more labile complex). Since k_{off} for the remaining sites should be similar to that determined in the native conformation (magnesium is assumed to occupy the same binding sites in each case), the overall effect should be to reduce ΔH^*, resulting in a shallower initial slope in the temperature profile. This is not observed. Furthermore, the inclusion of an additional set of weaker binding sites in the analysis routine (i.e., taking 50 sites with $K_a \sim 200$ and 25 with K_a in the range 1–20) does not improve the fit to the data. Although there is undoubtedly some variation in the exact values for each K_a and ΔG^* for any individual site, our evidence indicates that these are relatively small and are unlikely to have any biological significance. The weak sites in the grooves of the α-helices are fairly uniform and no sharp diversions in binding properties would be expected. A possible exception to this is discussed below.

It is not clear that the similarity between the number of major binding sites (ca. 82) in the nonnative conformation and the number of backbone phosphates (ca. 76), suggestive of a 1:1 binding ratio, is anything other than a fortuitous occurrence. The absence of strong binding sites in the nonnative structure presumably results from the repulsion between chains following the loss of the stabilizing influence of background Na^+. In the native state magnesium stabilizes tertiary structure by cross-linking adjacent bases in loop regions: binding to the backbone phosphates on U8/A9/C11/U12 (a in Figure 3) and G20/A21 (b in Figure 3), and bridging two single-strand regions by binding to G19, and hydrogen bonding via the solvent hydration shell to oxygens and nitrogens on U59, C60, and G20 (c in Figure 3) (Jack et al., 1976, 1977; Quigley et al., 1978). A further Mg^{2+} site has been putatively identified in the anticodon arm with direct binding to phosphate-38 and hydrogen bonding through water molecules to Y37, A38, Ψ39, and C32 by Quigley et al. (1978). It is difficult to say whether this is a strong or weak site, but it may be implicated with a special role with regard to ribosome function (see discussion below). One further possible weak site has been identified around residues 52, 53, and 58 (Quigley et al., 1978).

The absence of cooperativity in the native structure presumably indicates that the packing of the RNA chains is already optimal in the native structure and the counterions serve to stabilize this arrangement. In the nonnative conformation, however, the first few magnesium centers apparently bind in a cooperative manner, presumably around the loop regions at the turning points of the α-helices where the more stable cross-linking sites are located (Figure 3). The stabilization of these regions will then facilitate further binding. Subsequently, magnesium ions occupy sites in the grooves of the α-helices. Since there is little structural difference between these sites in the native and nonnative states, similar binding constants are observed. A number of denaturable tRNAs have been shown to exist in only two conformations, depending on salt conditions, and they have been analyzed in some detail. In 0.1 M NaCl, $tRNA^{Glu_2}$ exists as an equilibrium mixture of the two states and Stein and Stein (1976) have demonstrated using the binding isotherm of Monod et al. (1965) that ca. 8 Mg^{2+} are required to completely convert nonnative tRNA to the native state. This is in good qualitative agreement with our NMR data (Figure 1b) that indicates ca. 12. The discrepancy between this number and the number of strong binding sites in the native state was also noted by these authors, although no explanation was offered. We believe the most straightforward explanation demands that the native state requires not only the strong sites to be occupied but also a small number of additional weaker sites that may be differentiated from general binding in the grooves of the α-helices. Such sites could probably have a K_a up to an order of magnitude larger than the more general weak sites since it would be difficult to distinguish these by NMR, or in fact by any other method. Interestingly, Quigley et al. (1978) identified one additional Mg^{2+} ion at the anticodon loop, which was assigned as a weakly bound ion. If we take the analysis further, then this might suggest that previously "differing" ideas on the binding and release mechanisms of tRNA interaction with ribosomes, one invoking allosteric control (Stein & Stein, 1976) and the other in terms of codon–anticodon interaction (Kurland et al., 1975), might in fact be a result of a common mechanism requiring weak Mg^{2+} binding to the anticodon stem.

In comparison to smaller phosphate-bearing molecules, the K_a's for Mg^{2+} binding to tRNA in both native and nonnative conformations are large, particularly since the tRNA backbone is a phosphodiester and should bind less strongly than a phosphate. The large value of K_a presumably reflects multiple additional contacts with ribose hydroxyls and O/N atoms on bases, which have been crystallographically characterized for the strong binding domains (Jack et al., 1977). A more detailed analysis of the coordination chemistry of these sites will be presented elsewhere (Cowan, unpublished results). The similarity in χ_B between native and nonnative conformations (0.84 and 0.64, respectively) indicates the absence of change in the coordination environment. Previous determinations for Mg^{2+} binding to a number of proteins (troponin c, $\chi_B \sim 1.1$ MHz; calmodulin and tryptic fragments, $\chi_B \sim 1.6$ MHz) (Tsai et al., 1987; Forsen et al., 1983) reflect larger asymmetry. The retention of most of the waters of hydration (five or six) for Mg^{2+} binding to RNA should be compared with the coordination environment in proteins where typically several waters are lost following binding by chelating protein residues.

We note at this point that the kinetics of magnesium binding are also quite distinct from that observed for smaller phosphate-bearing molecules (Cowan, unpublished results). It is clear from the variable-temperature plots that the interaction of magnesium with tRNA lies in the slow-exchange region. The off-rates ($k_{off} = 2.5 \times 10^3$ s^{-1} and 1.6×10^3 s^{-1}) estimated from the activation free energies (eq 2) are similar, since the average activation enthalpies (ΔH^*) for each magnesium site (2.7 ± 0.2 kcal and 2.9 ± 0.2 kcal for native and nonnative, respectively) are almost identical under native and nonnative conditions. The cooperative behavior observed in the early part of Figure 1b points to a small subset of distinct binding sites analogous to the native conformation. However, these did not appear to influence the variable-temperature behavior of the line width. When the line-shape analysis was repeated assuming 6–12 additional strong binding sites with a range of association constants (500–10^4 M^{-1}), the resulting fit was always poor. Such strong sites as may exist in the nonnative conformation after the initial addition of ≥12 ions presumably adopt a native coordination mode and bind too strongly to be of significance in this VT experiment.

There are few direct methods for the study of the solution chemistry of magnesium ion with small ligands or biological complexes. Given the correct hardware and software, ^{25}Mg NMR represents a direct and, relative to other indirect methods, rapid manner of determining kinetic and thermodynamic parameters for metal ion binding to biological substrates and macromolecules and also a means of addressing questions of cooperativity. In the case of strong binding the

number of ions can also be directly determined by titration. These ions may then be functionally implicated as a trigger of biochemical response (Linse et al., 1987; Tsai et al., 1987). These data represent the first *direct* determination of the kinetics and thermodynamics of magnesium binding to RNA and quantitation of the number of bound ions. Neither the general issue of how the weakly bound ions interact with the backbone (inner- or outer-sphere coordination) nor the specific role of alkali metal ions has been discussed. These topics are important and are under investigation.

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