Nucleoside Modifications Stabilize Mg^{2+} Binding in *Escherichia coli* $tRNA^{Val}$: An Imino Proton NMR Investigation†

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ABSTRACT: The structures of *in vitro* transcribed *Escherichia coli* $tRNA^{Val}$, which lacks base modifications, and the native tRNA, which contains them, are very similar in the presence of excess Mg^{2+} (Kintanar, Yue, and Horowitz, unpublished results). To further probe the effects of base modifications on the structure of tRNA, the Mg^{2+} ion dependence of the downfield region of the ^1H NMR spectrum of *in vitro* transcribed *E. coli* $tRNA^{Val}$ in aqueous phosphate buffer was investigated. The spectra indicate a remarkable conformational change in unmodified *E. coli* $tRNA^{Val}$ coincident with binding or release of Mg^{2+}. Assignment of the imino proton resonances in the low Mg^{2+} form of the tRNA transcript allows a detailed description of the conformational change. There is near total disruption of the D stem and tertiary interactions in the absence of bound Mg^{2+}. A new strong interaction between the U67–A6 base pair and the G50–U64 wobble pair is observed, indicating a substantial structural rearrangement at the junction of the acceptor and T stems. The binding constants of the strong Mg^{2+} binding sites in the D loop and near the D stem in unmodified $tRNA^{Val}$ are at least 2 orders of magnitude less than in $tRNA^{Val}$ containing base modifications. The metal ion binding site in the anticodon loop is somewhat stronger than metal ion binding sites in the D loop and stem in unmodified $tRNA^{Val}$, but it is still weaker than all strong Mg^{2+} binding sites in native $tRNA^{Val}$. Thus, one role of the base modifications found in tRNA is to stabilize or strengthen the Mg^{2+} binding sites. The temperature dependence of the imino proton spectrum of *in vitro* transcribed *E. coli* $tRNA^{Val}$ in the absence of added Mg^{2+} was also investigated. In the low Mg^{2+} form of the tRNA, the interaction between U67–A6 and G50–U64 as well as the rest of the T stem remains intact and forms a stable structural core even at 60 °C. This structural core appears to be more stable than the anticodon stem, which is only partly intact at 60 °C.

One of the most striking characteristics of tRNA molecules is their large proportion (10–20%) of post-transcriptionally modified bases. Recently, it has become possible to synthesize tRNA *in vitro* using T7 RNA polymerase to transcribe a suitable DNA template (Sampson & Uhlenbeck, 1988). These *in vitro* transcribed tRNA molecules do not have the base modifications found in native tRNA, yet they are still efficiently aminoacylated by their respective cognate synthetases (Sampson & Uhlenbeck, 1988; Chu & Horowitz, 1989). The implication is that the structure of the *in vitro* transcript is similar to that of native tRNA.

In support of this, the downfield (imino proton region) ^1H NMR spectra of both modified and unmodified *Escherichia coli* $tRNA^{Val}$ are very similar when there is an excess of Mg^{2+} ion in solution (Kintanar, Yue, and Horowitz, unpublished results). These results indicate that the structure of the *in vitro* transcript is very similar to that of the native tRNA molecule at high Mg^{2+} concentrations. A similar conclusion was reached by Hall *et al.* (1989) working with unmodified yeast phenylalanine tRNA.

The structural stability of *in vitro* transcribed tRNA molecules is, however, less than that of the native tRNA molecule. This is reflected in the melting temperatures (T_m), which are invariably lower for the transcript than for the corresponding base-modified tRNA (Sampson & Uhlenbech, 1988; Derrick & Horowitz, 1993), and in the ^{19}F NMR spectrum of *in vitro* transcribed 5-fluorouracil substituted *E. coli* $tRNA^{Val}$, which indicates a disordered structure of parts of the unmodified tRNA under conditions of low Mg^{2+} concentration (Chu & Horowitz, 1989).

Previously, Hall *et al.* (1989) investigated the effect of Mg^{2+} concentration on the structure of *in vitro* transcribed yeast phenylalanine tRNA. They found that the unmodified tRNA adopted a "semidenatured" conformation and reported evidence for the formation of an additional G–U base pair at low Mg^{2+}, but further details of the altered conformation were unavailable. Thus, one possible role for base modifications in tRNA is to increase the stability of the structure by a mechanism that may be linked to the binding of Mg^{2+} to the tRNA (Schimmel & Redfield, 1980; Guenther *et al.*, 1992; Dao *et al.*, 1992; Chen *et al.*, 1993).

In this paper, we probe this hypothesis more deeply by observing the effect of temperature and Mg^{2+} concentration on the downfield ^1H NMR spectrum of *in vitro* transcribed *E. coli* $tRNA^{Val}$. To accurately follow the Mg^{2+} titration, we have confirmed the assignments of the ^1H spectrum under conditions of low Mg^{2+} concentration using one-dimensional difference NOE[1] methods. The assigned imino proton NMR spectrum provides excellent low-resolution structural reporters of the effects of temperature and Mg^{2+} concentration.

MATERIALS AND METHODS

DNA templates for *in vitro* transcription by T7 RNA polymerase were derived from the recombinant phagemid

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy.

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pVAL119-21, containing the cloned wild-type *E. coli* $tRNA^{Val}$ gene linked directly to an upstream bacteriophage T7 promoter, as described previously (Chu & Horowitz, 1989). Transcription reactions were performed as described previously (Chu & Horowitz, 1989) using T7 RNA polymerase prepared according to the method of Grodberg and Dunn (1988). The transcript was purified by high-pressure liquid chromatography (HPLC) as described previously (Chu & Horowitz, 1989). Purified tRNA was precipitated by addition of ethanol, washed with 70% ethanol, and dried.

For ^1H NMR spectroscopy, 5–25 mg of tRNA was dissolved in a minimal amount of buffer containing 11 mM sodium phosphate, pH 7.0, 16.5 mm $MgCl_2$, and 111 mM NaCl and dialyzed against two to three changes of 250 mL of the same buffer. The sample volume was adjusted to 405 μL with dialysis buffer, and 45 μL of D_2O was added. The sample for Mg^{2+} titration was prepared with no added Mg^{2+} by exhaustive dialysis against 11 mM sodium phosphate, pH 7.0, prior to final volume adjustment. Some samples with no added Mg^{2+} were also prepared by first dialyzing against 10 mM EDTA followed by heating to 70 °C and then dialyzing against the phosphate buffer to remove excess EDTA. Samples of unmodified tRNA with no added Mg^{2+} prepared according to either method could not be distinguished by ^1H NMR spectroscopy. Titration with Mg^{2+} was effected by adding small aliquots of concentrated $MgCl_2$ solutions (0.2–1.0 M) directly to the NMR tube. Native *E. coli* $tRNA^{Val}$ was purchased from Subriden RNA (Seattle, WA) and prepared without added Mg^{2+} by exhaustive dialysis against phosphate buffer.

All ^1H NMR spectroscopy was performed at 500 MHz on a Varian Instruments Unity-500 NMR spectrometer. One-dimensional NMR spectra were collected with the H_2O resonance suppressed using the 1–1 spin–echo selective excitation pulse sequence (Sklenar & Bax, 1987). Typical parameter settings and data processing were as described previously (Kintanar *et al.*, 1991), except the spectra were also sometimes apodized with a shifted sine bell (~35°) to enhance resolution. A typical sample containing ~7 mg of tRNA required about 20 min of data acquisition (several hundred scans) to obtain spectra with good signal-to-noise ratios.

One-dimensional difference NOE experiments were performed with a pulse sequence consisting of a 100-ms presaturation pulse followed by a jump–return pulse (Plateau & Guéron, 1982) to suppress the H_2O resonance. The presaturation pulse was of sufficient power to saturate the irradiated peak by ~90%. The NOE data were obtained by taking the difference between the spectrum collected with the presaturation pulse centered on the peak of interest and a control spectrum collected with the presaturation pulse set off-resonance, well downfield. Typically, 3000 scans were collected at each frequency offset. The delay between the pulses of the jump–return sequence was typically 40 μs to allow good excitation of the downfield imino resonances. Other spectral and data processing parameters were similar to those for 1–1 echo one-dimensional ^1H NMR experiments.

The temperature of the NMR sample was regulated by heated (or cooled) air flow and was measured by thermocouple near the sample coil. The temperatures were accurate to ±0.5 °C. The H_2O peak served as the chemical shift reference and was assumed to resonate at 4.80 ppm at 22 °C, and to shift upfield by 5 Hz/°C with increasing temperature.

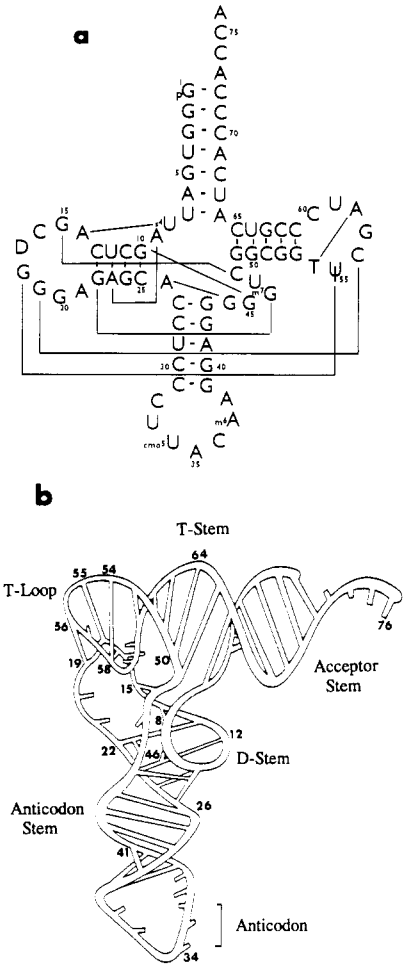

FIGURE 1: Schematic models of the primary, secondary, and tertiary structures of *E. coli* $tRNA^{Val}$: (a) cloverleaf model; (b) model of the tertiary molecular structure at low resolution.

RESULTS

The secondary structure and expected tertiary interactions of $tRNA^{Val}$ are shown in Figure 1. Imino protons that are involved in hydrogen bonds in base pairs or tertiary interactions are protected from exchange with solvent (Kearns *et al.*, 1971). These imino protons are visible in the downfield region of the ^1H NMR spectrum between 11 and 15 ppm. On the basis of the model shown in Figure 1, which was derived from the crystal structure of yeast phenylalanine tRNA (Kim *et al.*, 1974; Robertus *et al.*, 1974), 28 imino protons are protected from exchange by hydrogen bonding between bases and should be visible in the downfield ^1H NMR spectrum. Additionally, imino protons that are involved in hydrogen bonds to the backbone might also be expected to contribute to the downfield imino spectrum (downfield of 10 ppm), as might strongly hydrogen bonded amino protons (Smith & Feigon, 1993).

Imino Proton Spectrum at Low Mg^{2+}. The downfield ^1H NMR spectra of native and *in vitro* transcribed *E. coli* $tRNA^{Val}$ with no added Mg^{2+} are shown in Figure 2. Unlike the case when there is excess Mg^{2+} in solution (Kintanar, Yue, and Horowitz, unpublished results), the spectra of the modified and unmodified tRNA are considerably different. In the spectrum of the *in vitro* transcript (Figure 2b), there are now only ~16 sharp peaks between 11 and 15 ppm—versus ~21 under conditions of excess Mg^{2+}—and the total intensity in these resonances corresponds to only ~18 protons—versus ~29 with excess Mg^{2+} (compare Figure 3f). Additionally, there are new broad resonances with considerable intensity

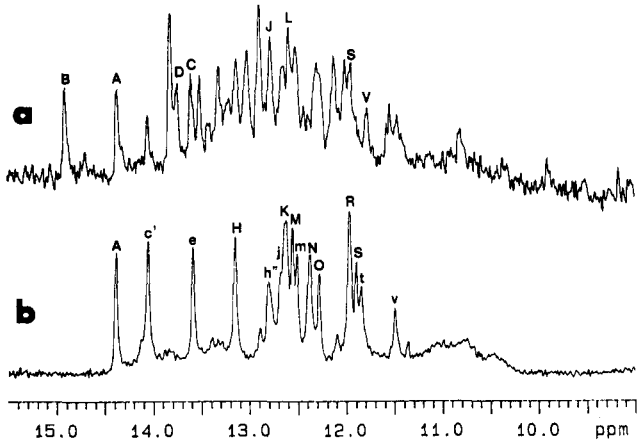

FIGURE 2: Expanded region of the ^1H NMR spectra of tRNA in H_2O (10% D_2O) with 10 mM sodium phosphate in the absence of added Mg^{2+} at 22 °C: (a) native *E. coli* $tRNA^{Val}$; (b) *E. coli* $tRNA^{Val}$ transcribed *in vitro*.

between 10 and 11 ppm, characteristic of imino protons not involved in hydrogen bonds (Hare & Reid, 1986). In contrast, the imino proton spectrum of native *E. coli* $tRNA^{Val}$ with no added Mg^{2+} (Figure 2a) is very similar to that obtained with excess Mg^{2+} (Kintanar, Yue, and Horowitz, unpublished results). The two spectra have about equal proton intensity and number of resolved resonances, and there are only small shifts of a few resonances between spectra.

The loss of intensity in the imino proton spectrum of the Mg^{2+}-deficient unmodified *E. coli* $tRNA^{Val}$ indicates that some imino protons have become accessible to solvent exchange, presumably due to a substantial change of tRNA conformation at low Mg^{2+} concentration. To verify which proton resonances have been broadened by solvent exchange, it is necessary to determine the assignments of the remaining resonances in the spectrum of the *in vitro* transcript with no added Mg^{2+}. This is readily accomplished using one-dimensional difference NOE methods and by following changes in the spectrum as Mg^{2+} is titrated into the sample.

Titration with Mg^{2+}. A representative series of ^1H NMR spectra of *in vitro* transcribed *E. coli* $tRNA^{Val}$ at different Mg^{2+} concentrations is shown in Figure 3. In Figure 3a, the molar ratio of Mg^{2+} to tRNA is 1, and the spectrum is essentially identical to that of the unmodified tRNA with no added Mg^{2+} (Figure 2b). In Figure 3f, the molar ratio of Mg^{2+} to tRNA is 40, and the spectrum is very similar to that of the unmodified tRNA in the presence of excess (15 mM free) Mg^{2+} (Kintanar, Yue, and Horowitz, unpublished results).

At intermediate molar ratios of Mg^{2+} to tRNA, there are significant changes to most of the resonances in the ^1H spectrum of *in vitro* transcribed *E. coli* $tRNA^{Val}$. The imino proton spectrum of native *E. coli* $tRNA^{Val}$ (with modified bases) has already been assigned (Hare *et al.*, 1985). The NMR resonance assignments of *in vitro* transcribed *E. coli* $tRNA^{Val}$ in the presence of excess Mg^{2+} are very similar except for the chemical shifts of resonances corresponding to exchangeable protons in, or interacting with, modified bases (Kintanar, Yue, and Horowitz, unpublished results). Therefore, it is easier to follow the Mg^{2+} titration in reverse, that is, from high to low Mg^{2+} concentration. As the Mg^{2+} concentration decreases, some peaks gradually lose intensity and disappear, others lose intensity and are replaced by new peaks that concomitantly grow in intensity, while others undergo very little change except for small shifts. The ^1H NMR spectra of *in vitro* transcribed *E. coli* $tRNA^{Val}$ at 13

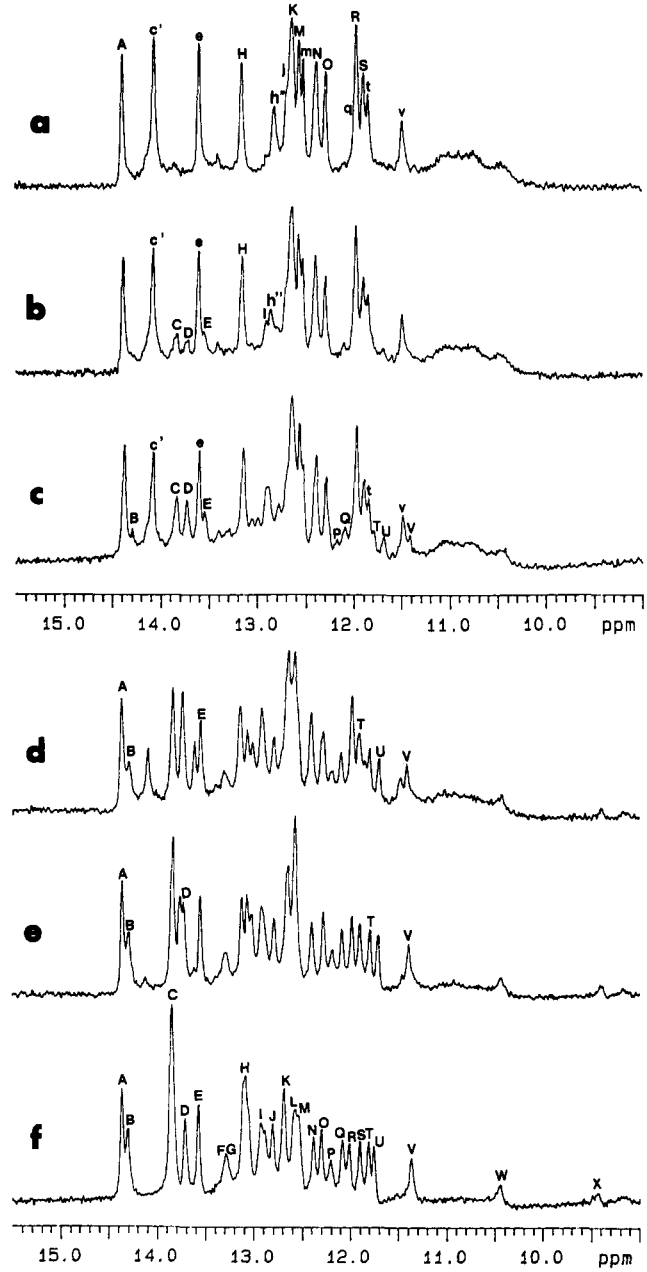

FIGURE 3: Mg^{2+} titration of unmodified *E. coli* $tRNA^{Val}$ shown by the downfield region of the ^1H NMR spectra of the *in vitro* transcript at 22 °C. The solution conditions were as in Figure 2 except Mg^{2+} was added to attain the indicated Mg^{2+}/tRNA ratio, γ: (a) $\gamma = 1$; (b) $\gamma = 4$; (c) $\gamma = 6$; (d) $\gamma = 10$; (e) $\gamma = 18$; (f) $\gamma = 40$.

different Mg^{2+} concentrations were obtained, and the results are summarized in Figure 4. In the following discussion, the peak labels (in Figures 3 and 4) refer to assigned resonances in the downfield ^1H NMR spectrum of *in vitro* transcribed *E. coli* $tRNA^{Val}$ in the presence of excess Mg^{2+} (Kintanar, Yue, and Horowitz, unpublished results). These resonance assignments are summarized in Table 1. New peaks that appear at lower Mg^{2+} concentrations are designated by lower case letters, which indicate a probable correlation to peaks at higher Mg^{2+} concentrations.

Peaks that undergo little change in shift or intensity and can be readily followed during the course of the titration are A (U67), H (G3), K (G2), N (G52), O (G40), R (G1), and S (U64). The imino protons to which these peaks are assigned are given in parentheses (Kintanar, Yue, and Horowitz, unpublished results). The schematic structural models in Figure 1 indicate the secondary or tertiary interactions that

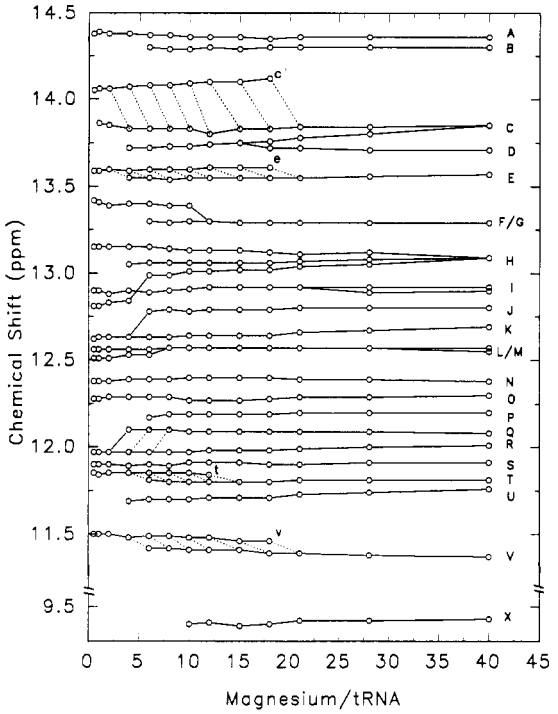

FIGURE 4: Plot of the chemical shift of the downfield ^{1}H NMR peaks as a function of the Mg^{2+}/tRNA ratio. A dotted line joining two points indicates that there are two resonances attributable to a particular proton, corresponding to two conformations (with and without bound Mg^{2+}) of tRNA. The disappearance of a solid line at a particular Mg^{2+}/tRNA ratio indicates the disappearance of a peak.

Table 1: Exchangeable Proton Assignments of *E. coli* $tRNA^{Val}$ with Excess Mg^{2+} Ion[a]

peak	assignment[b]	peak	assignment[b]
A	U67	K	G2
B	U8	K′	G46
C	U54	L	U7
C′	U29	M	G39
C″	U12	M′	G10
D	G24	N	G52
E	U4	O	G40
F	G44	P	G15
G	G43	Q	G49
H	G3	R	G1
H′	G53	S	U64
H″	G42	T	G5
I	G22	U	U55
I′	G19	V	G50
J	G63	X	G18

[a] Assignments are from Kintanar, Yue, and Horowitz (unpublished results). [b] All assignments are to imino protons except peak X, which is assigned to the G18 amino proton.

protect the imino protons from solvent exchange, thereby making the corresponding resonance detectable by ^{1}H NMR spectroscopy. Peaks that lose intensity but seem to grow in at new positions with decreasing Mg^{2+} concentration are C′ (U29), E (U4), H′ (G53), H″ (G42), J (G63), M (G39), O (G40), Q (G49), T (G5), and V (G50). Peaks that apparently lose all or most of their intensity are B (U8), C (U54), C″ (U12), D (G24), F (G44), G (G43), I (G22), I′ (G19), K′ (G46), L (U7), M′ (G10), P (G15), U (U55), W (unassigned), and X (G18 amino).

At many Mg^{2+} concentrations, there are two peaks corresponding to one imino proton (e.g., peaks E and e, M and m, T and t, V and v). This indicates that there are two conformations of the unmodified tRNA molecule in solution corresponding, presumably, to tRNA with and without a metal ion bound at a particular (nearby) site. The two conformations are in slow exchange with each other on the chemical shift time scale.

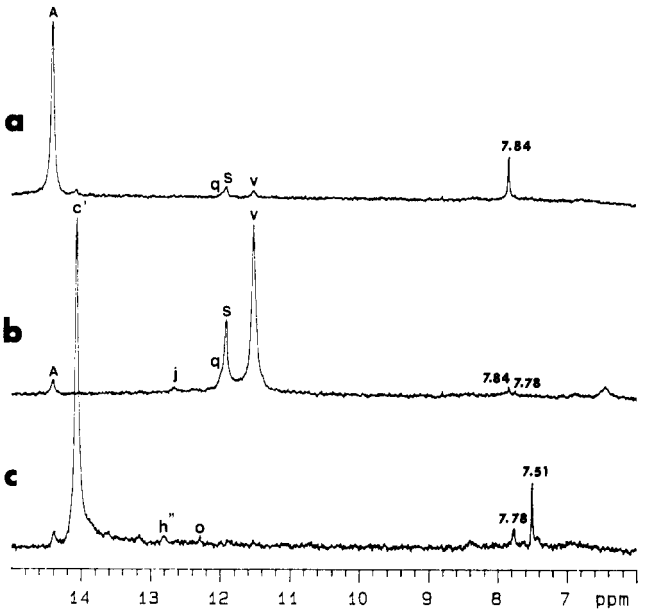

FIGURE 5: Downfield region of ^{1}H NMR one-dimensional difference NOE spectra of the unmodified tRNA at the same solution conditions and temperature as in Figure 2. (a) Peak A has been saturated and shows NOEs to imino peaks q, S, and v and to an aromatic peak at 7.84 ppm. (b) Peak v has been saturated and shown NOEs to imino peaks A, j, q, and S and to aromatic peaks at 7.84 and 7.78 ppm. (c) Peak c′ has been saturated revealing NOEs to imino peaks h″ and o and to an aromatic peak at 7.51 ppm. The apparent NOE at 7.78 ppm comes from excitation of peak 1, which under these conditions is broad and hidden under peak c′. Other apparent NOEs that have not been labeled are believed to be artifacts of power spillover or incomplete cancellation or else could not be unequivocally assigned.

NOE at Low Mg^{2+}. It is quite easy to misassign peaks when changes in intensity and position are followed during the course of a titration. The assignments have been confirmed, as much as possible, using one-dimensional difference NOE methods. In addition, the NOE data can give information about possible conformational changes of the unmodified tRNA at low Mg^{2+}. Although two-dimensional NOESY spectra have been shown to be a very effective method for obtaining imino proton assignments (Hare *et al.*, 1985; Hall *et al.*, 1989), and in fact we used these methods to assign the imino proton spectrum of *E. coli* $tRNA^{Val}$ at high Mg^{2+} concentration (Kintanar, Yue, and Horowitz, unpublished results), we found that the one-dimensional difference NOE methods sufficed for confirming the assignments and had the added advantage of requiring less sample and avoiding potential problems with aggregation. Some representative one-dimensional difference NOE spectra are shown in Figure 5. For example, upon irradiation of peak A, there is a strong NOE to a sharp resonance at 7.84 ppm which probably corresponds to a C2 proton (Figure 5a). This indicates that peak A corresponds to an imino proton in an A–U Watson–Crick base pair (U67) as expected from the previous assignment of peak A at high Mg^{2+} concentration (Kintanar, Yue, and Horowitz, unpublished results). The NOEs to peaks L (U7) and T (G5) that were observed for the unmodified tRNA at high Mg^{2+} (Kintanar, Yue, and Horowitz, unpublished results) are not seen. Peak L appears to have disappeared at 22 °C, but there is evidence for a peak that may correspond to the U7 imino proton at lower temperature (see below).

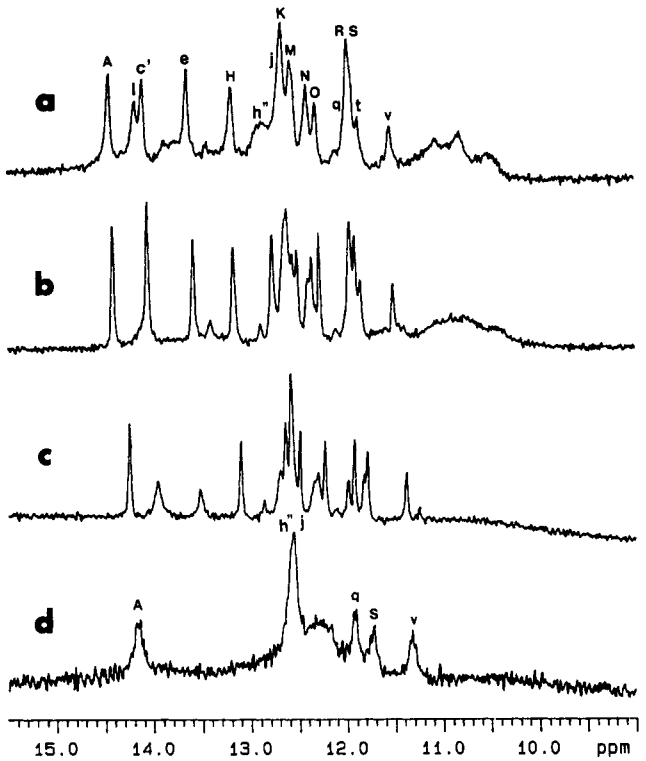

FIGURE 6: Expanded region of ^1H NMR spectra of unmodified *E. coli* $tRNA^{Val}$ obtained at different temperatures: (a) 8 °C; (b) 22 °C; (c) 47 °C; (d) 60 °C. The solution conditions were as in Figure 2. The labeled peaks are assigned as follows: A (U67); h″ (G42); j (G63); Q (G49); S (U64); v (G50).

The NOEs observed generally support the NMR resonance assignments for the *in vitro* transcribed *E. coli* $tRNA^{Val}$ at low Mg^{2+} concentration derived from following the effects of Mg^{2+} titration and the NMR resonance assignments of *in vitro* transcribed *E. coli* $tRNA^{Val}$ in the presence of excess Mg^{2+} (Kintanar, Yue, and Horowitz, unpublished results).

Effects of Temperature at Low Mg^{2+}. The ^1H NMR spectra of *in vitro* transcribed *E. coli* $tRNA^{Val}$ at low Mg^{2+} concentration at four temperatures are shown in Figure 6. The spectrum in Figure 6b was obtained at the same temperature, 22 °C, used for the assignment of the imino proton spectrum of the low Mg^{2+} form (see above) and is essentially identical to that shown in Figure 2b. At 47 °C (Figure 6c), there are shifts of several resonances, some resonances lose intensity, and some resonances become broader. It is easy to follow the resonances with temperature by observing a series of temperatures and determining the temperature coefficient of the shift of the hydrogen-bonded imino protons. The temperature coefficient of the imino proton shift is assumed to be constant over a narrow temperature range in the absence of conformational changes of the molecule. Resonances that broaden and lose intensity are c′ (U20) and e (U4), which correspond to protons involved in generally more labile U–A Watson–Crick base pairs. Peaks R (G1) and t (G5) lose intensity without broadening. Peaks that indicated two conformations of tRNA in slow exchange, M (G39) and O (G40), indicate only one conformation at elevated temperature. At 60 °C (Figure 6d), only resonances due to imino protons in the T stem, some of the C–G base pairs of the anticodon stem (except peak G, G43), and one A–U base pair (peak A, U67) in the acceptor stem remain. The observation of a high-temperature A–U base pair sharp resonance is somewhat unusual. To verify this, a one-dimensional difference NOE experiment was performed by irradiating peak A at 53 °C. A single strong NOE to an apparent sharp C2H resonance in the aromatic region was observed (data not shown), confirming the identity of peak A as a U imino proton (U67) in a Watson–Crick A–U base pair.

At 8 °C (Figure 6a), a new resonance appears, just downfield of peak c′. This resonance is probably an imino proton on a Watson–Crick A–U base pair because a strong NOE to an apparent sharp C2H resonance in the aromatic region is observed at 8 °C (data not shown). The C2H resonance corresponds to the previously unassigned NOE peak at 7.78 ppm, which was observed upon irradiation of peak c′ at 22 °C (Figure 5c). The unassigned low-temperature imino proton resonance is broad and shifted under peak c′ at 22 °C. The candidates for the low-temperature imino proton resonance are peak c″ (U12), peak l (U7), or a new U–A Watson–Crick base pair that is forming in the altered conformation of tRNA at low Mg^{2+}. At low Mg^{2+}, no resonances or NOEs are observed that could be attributed to D stem imino protons, making c″ (U12) an unlikely assignment. Peak l (U7) seems a reasonable assignment, but a confirming NOE to peak A (U67) could not be observed because of spillover problems. Upon irradiation of peak v (G50) at 22 °C, however, there is a very weak NOE at 7.78 ppm (Figure 5b). The NOE observed between peaks A and v establishes the proximity of base pairs G50–U64 and A6–U67 in the unmodified tRNA at low Mg^{2+} concentration. The base pair U7–A66, if it is intact, might also be close to G50–U64. Thus, the apparent C2H resonance at 7.78 ppm may correspond to the A66 C2H, and the associated imino resonance, observed downfield of c′ at 8 °C, may be tentatively assigned to the U7 imino proton (peak l).

DISCUSSION

Mg^{2+}-Dependent Conformational Change. The ^1H NMR spectrum of *in vitro* transcribed *E. coli* $tRNA^{Val}$ shows a strong dependence on the Mg^{2+} concentration. In particular, at a Mg^{2+} to tRNA molar ratio below one, there is a loss of at least 10 resonances from the spectrum (Figure 1b) compared to that obtained at high Mg^{2+} concentration. At an identical low Mg^{2+} concentration, the spectrum of native (modified) *E. coli* $tRNA^{Val}$ (Figure 2a) is little different from that at high Mg^{2+}/tRNA ratios. This indicates a dramatic conformational change of the unmodified tRNA molecule at low Mg^{2+} concentration which renders several imino protons more susceptible to solvent exchange. The conformational change includes significant disruption of the secondary and tertiary structure so the molecule is less compact and there are fewer imino protons involved in hydrogen bonds. The tRNA molecule is in a semidenatured state. In native (modified) tRNA, assembly of the native (active) tertiary structure has been shown to be associated with cooperative binding of Mg^{2+} (Schimmel & Redfield, 1980). These effects were observed, however, at much lower Mg^{2+} concentrations ($\sim 10\ \mu M$) than are required to effect conformational changes in the unmodified tRNA molecule.

By assigning the ^1H NMR spectrum of the unmodified tRNA at low Mg^{2+}, we are able to understand the changes to the spectrum and to the structure of tRNA as the Mg^{2+} concentration is lowered. At low Mg^{2+} concentration, we observe resonances and NOEs arising from the acceptor stem, the T stem, and the anticodon stem. The D stem is however, disrupted; we do not find any resonances or NOEs that would convincingly indicate the presence of an intact D stem.

Additionally, we do not observe any resonances or NOEs that would strongly indicate the presence of tertiary interactions in the *in vitro* transcribed *E. coli* $tRNA^{Val}$ at low Mg^{2+}.

Several observations indicate a substantial conformational change of unmodified *E. coli* $tRNA^{Val}$ at the junction of the acceptor and T stems under conditions of low Mg^{2+} concentration. We observe new NOEs between the imino proton resonance of U67 (peak A) and the imino proton resonances of G50 (V), U64 (S), and G49 (Q) at low Mg^{2+} concentration, suggesting that the A6–U67 base pair is stacked more directly on the G50–U64 base pair than on the G49–C65 base pair. Moreover, at 22 °C or above, there is little or no intensity of peaks assignable to the U7 imino proton, suggesting that the U7–A66 base pair has decreased thermal stability at low Mg^{2+} concentrations. In both modified and unmodified *E. coli* $tRNA^{Val}$ at high Mg^{2+} concentration, the peak corresponding to the U7 imino proton is found at an unusual upfield position (Hare *et al.*, 1985; Kintanar, Yue, and Horowitz, unpublished results). The precise origin of this upfield shift is not understood, although it is presumably due to a unique structural feature at the junction of the acceptor and T stems. In the unmodified *E. coli* $tRNA^{Val}$ at low Mg^{2+} concentration, a peak tentatively assigned as l (U7) is found just downfield of c′ at 8 °C. No peak assignable to the U7 imino proton is found at a more upfield position. Thus, the structural feature causing the upfield shift of the U7 imino proton resonance is apparently not present in the unmodified tRNA at low Mg^{2+} concentrations.

Binding of Mg^{2+}. It is of interest to consider the conformational changes of the unmodified *E. coli* $tRNA^{Val}$ at low Mg^{2+} concentrations, in light of what is known about the Mg^{2+} binding sites. There are both strong and weak Mg^{2+} binding sites (Schimmel & Redfield, 1980), and the location of the strongest binding sites in yeast phenylalanine tRNA can be determined from the X-ray crystal structure (Jack *et al.*, 1977; Holbrook *et al.*, 1977; Quigley *et al.*, 1978). The four strong Mg^{2+} binding sites seen in the crystal structure of yeast phenylalanine tRNA are in the D loop (two), near the D stem, and in the anticodon loop (Holbrook *et al.*, 1977; Quigley *et al.*, 1978). One of the bound metal ions in the D loop appears to stabilize tertiary interactions between the D and T loops, while the bound metal ion near the D stem appears to stabilize the turn from U8 to A14 (P10 loop) and associated tertiary interactions (Quigley *et al.*, 1978). Given that most of the invariant residues in class I tRNAs are located near the Mg^{2+} binding sites, it is reasonable to assume that *E. coli* $tRNA^{Val}$ has strong Mg^{2+} binding sites at locations similar to those in yeast phenylalanine tRNA.

The disruption of the secondary and tertiary structure of *in vitro* transcribed *E. coli* $tRNA^{Val}$ that we observe at low Mg^{2+} concentrations is consistent with the loss of bound metal at the D loop and the D stem, resulting in the loss of tertiary interactions between the D and T loops and a disruption of the D stem. The loss of these interactions releases some conformational restraints, and the resulting relaxation of the structure alters the conformation at the junction of the T and acceptor stem. In native *E. coli* $tRNA^{Val}$, we do not observe these conformational changes under similar low Mg^{2+} concentrations. This indicates that the Mg^{2+} ions at the four sites are bound more tightly to the modified *E. coli* $tRNA^{Val}$ than to the molecule without base modifications. Much harsher treatments (e.g., heating in the presence of EDTA) are required to strip the Mg^{2+} bound to these sites and disrupt the secondary and tertiary structure in the native tRNA molecule (Lynch & Schimmel, 1974). Thus, one role of the base modifications is to strengthen or stabilize the Mg^{2+} binding sites. The previous ^1H NMR study of *in vitro* transcribed yeast phenylalanine tRNA supports this finding (Hall *et al.*, 1989). Studies of the yeast phenylalanine tRNA anticodon stem–loop region (Chen *et al.*, 1993) and of a DNA analog (Dao et al., 1992) also indicate that modification of a cytosine base in the stem region facilitates site-specific Mg^{2+} binding, which in turn induces a conformational change in the anticodon loop.

We observed two slowly exchanging conformations of the unmodified tRNA molecule in solution at intermediate Mg^{2+} concentrations. Two resonances, M (G39) and O (G40), indicate there are two conformations (with and without bound metal) near the anticodon loop even with no added Mg^{2+}. Other resonances indicating two conformers reveal a shift to only one, the form without bound metal, below a Mg^{2+} to tRNA molar ratio of six. Peaks losing intensity with decreasing Mg^{2+} concentration seem to have lost most of their intensity below a Mg^{2+} to tRNA molar ratio of six. It seems clear that the Mg^{2+} binding site closest to the G39 and G40 imino protons, in the anticodon loop, retains bound metal ion even at the lowest Mg^{2+} concentration investigated, i.e., no added Mg^{2+}. The other resonances report less than ~10% of tRNA molecules contain bound metal at nearby sites when the Mg^{2+} to tRNA molar ratio is less than six. Thus, the metal ion binding sites in the D loop and near the D stem appear to be considerably weaker than the site in the anticodon loop in *in vitro* transcribed *E. coli* $tRNA^{Val}$. From the ^1H NMR spectra at intermediate (known) Mg^{2+} concentrations and the known concentration of tRNA in our sample, and neglecting cooperative binding effects, we may obtain a very crude estimate of the association constant K_{assoc} of Mg^{2+} binding at a particular site. This estimation makes use of the relative proportion of the metal-free and metal-bound conformations of tRNA which can be determined from the heights of the resonances corresponding to the respective conformations. For example, the relative height of peaks e and E (U4) in Figure 3c (6-fold molar excess of Mg^{2+}) is about 3:1, indicating that the metal binding sites near the acceptor stem are only 25% occupied. For the anticodon loop binding site, we estimate a K_{assoc} of $\sim 1 \times 10^{-4}$ M^{-1}, and for the other metal binding site, we estimate a K_{assoc} of ~50. These estimated association constants are considerably less than those found for the strong Mg^{2+} binding sites in tRNAs containing modified bases, which are in the range of 1×10^5 M^{-1} (Schimmel & Redfield, 1980).

The rate of exchange between the metal-free and metal-bound conformations of tRNA is also worthy of comment. The exchange rate must be much smaller than the smallest frequency difference between resolved resonances corresponding to the two conformations ($k_{ex} \ll 2\pi\Delta\nu$). We observe resolved resonances that are 10 Hz apart. Thus, we estimate the conformational exchange rate to be $\ll 60$ s^{-1}. This slow rate is consistent with the requirement of a significant conformational change upon binding or release of Mg^{2+}. In a study of the cooperative binding of Mg^{2+} to native (modified) tRNA using a fluorescent probe, Lynch and Schimmel (1974) determined the conformational exchange rate to be less than 0.25 s^{-1}.

In a previous study of *in vitro* transcribed yeast phenylalanine tRNA, Hall *et al.* (1989) obtained only partial assignments of the downfield ^1H NMR spectrum in the absence of Mg^{2+}. Here, we are able to report more complete assignments of the downfield ^1H NMR spectrum of unmodified *E. coli* $tRNA^{Val}$ without added Mg^{2+}. This is probably just

a reflection of the somewhat better spectral resolution of our sample. Nevertheless, it enables us to give a more complete description of the "semidenatured" state of the unmodified tRNA molecule at low Mg^{2+} concentration (*vide supra*). Hall and co-workers (Hall *et al.*, 1989) observed the formation of a second G–U base pair in their unmodified tRNA molecule in the absence of Mg^{2+} and speculated that this could be a modified interaction between G18 and U55. We find no evidence for a second G–U base pair in unmodified $tRNA^{Val}$ at low Mg^{2+} concentration. In fact, base pairing between G18 and U55 is unlikely in light of our results showing the disruption of D loop to T loop tertiary interactions under these conditions. The absence of a second G–U base pair in the low Mg^{2+} form of unmodified *E. coli* $tRNA^{Val}$ may reflect differences in the primary sequences of the two tRNA molecules.

Temperature Dependence at Low Mg^{2+}. At room temperature and at low Mg^{2+} concentration, the D stem and tertiary interactions are totally disrupted. As the temperature is raised, the acceptor stem is disrupted with the exception of the U67–A6 base pair (peak A). The unusually high temperature stability of this A–U base pair is due to the altered conformation of unmodified tRNA at low Mg^{2+} concentrations. The U67–A6 base pair interacts strongly with the G50–U64 wobble base pair under conditions of low Mg^{2+}, and this interaction presumably stabilizes both base pairs. At 60 °C, some resonances of imino protons in the anticodon stem remain, but they are substantially broader than imino resonances of the T stem and the U67–A6 base pair. The T stem and the strongly associated U67–A6 base pair forms a thermally stable structural core in unmodified tRNA at low Mg^{2+} concentration.

In summary, this work reveals an altered structure for *in vitro* transcribed *E. coli* $tRNA^{Val}$ under conditions of low Mg^{2+} concentration. We have shown that there is a disruption of the D stem and the loss of tertiary interactions and that these conformational changes may be correlated to the decreased strength of Mg^{2+} binding, particularly at sites in the D loop and stem. The decreased Mg^{2+} binding ability is presumably due to the absence of base modifications. Given the low-resolution structural information available from this work, we cannot elaborate on the mechanism by which the weakened Mg^{2+} binding is effected. The lower thermal stability of unmodified tRNA at low Mg^{2+} concentrations is a consequence of the absence of bound Mg^{2+} leading to weaker tertiary interactions and a less compact semidenatured molecular conformation.

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