**NMR structure and Mg\(^{2+}\) binding of an RNA segment that underlies the L7/L12 stalk in the *E.coli* 50S ribosomal subunit**

Qin Zhao\(^1\), Uma Nagaswamy\(^1\), Hunjoong Lee\(^2\), Youlin Xia\(^2\), Hung-Chung Huang\(^1\), Xiaolian Gao\(^1,2\) and George E. Fox\(^1,\ast\)

\(^1\)Department of Biology and Biochemistry and \(^2\)Department of Chemistry, University of Houston, Houston, TX 77204-5001, USA

Received February 11, 2005; Revised and Accepted May 10, 2005

**ABSTRACT**

Helix 42 of Domain II of *Escherichia coli* 23S ribosomal RNA underlies the L7/L12 stalk in the ribosome and may be significant in positioning this feature relative to the rest of the 50S ribosomal subunit. Unlike the *Haloarcula marismortui* and *Deinococcus radiodurans* examples, the lower portion of helix 42 in *E.coli* contains two consecutive G•A oppositions with both adenines on the same side of the stem. Herein, the structure of an analog of positions 1037–1043 and 1112–1118 in the helix 42 region is reported. NMR spectra and structure calculations support a *cis* Watson–Crick/Watson–Crick (cis W.C.) G•A conformation for the tandem (G•A)\(_2\) in the analog and a minimally perturbed helical duplex stem. Mg\(^{2+}\) titration studies imply that the cis W.C. geometry of the tandem (G•A)\(_2\) probably allows O6 of G20 and N1 of A4 to coordinate with a Mg\(^{2+}\) ion as indicated by the largest chemical shift changes associated with the imino group of G20 and the H8 of G20 and A4. A cross-strand bridging Mg\(^{2+}\) coordination has also been found in a different sequence context in the crystal structure of *H.marismortui* 23S rRNA, and therefore it may be a rare but general motif in Mg\(^{2+}\) coordination.

**INTRODUCTION**

Helix 42 in Domain II of the *Escherichia coli* 23S ribosomal RNA (rRNA) (Figure 1a), is the main connecting element between the L7/L12 stalk and the rest of the 50S ribosomal subunit (1). The L7/L12 stalk encompasses the GTPase-associated domain of the 23S rRNA, which has interactions with ribosomal proteins L10, L7/12 and L11 (2–6). The L7/L12 stalk is well known to be dynamic, adopting alternative conformations at different stages in the elongation cycle (7–9). If bending in helix 42 occurred, it would have significant effect on the relative orientation of the L7/L12 stalk and the rest of the 50S subunit. In *E.coli* 23S rRNA, the lower portion of helix 42 contains two tandem G•A juxtapositions (1039/1116 and 1040/1115) in which both adenines are on the same side of the stem. These G•A oppositions are replaced by A–U and G•U in *Haloarcula marismortui*, and U–A and G–C in *Deinococcus radiodurans*. The 50S subunit crystal structures from these other organisms reveal that in both cases, the corresponding bases are paired and the entire stem is a standard A-form helix. In addition, there are no ribosomal proteins in close proximity. Examination of 23S rRNA sequences from other organisms, e.g. *Thermus thermophilus*, *Zea mays* chloroplast and *Bacillus stearothermophilus* revealed that normal Watson–Crick pairs frequently occur in positions corresponding to the tandem (G•A)\(_2\) interactions found in *E.coli* (10).

G•A mismatch pairs occur very frequently in RNA and actually are comparable in number in the large ribosomal RNAs to G–U wobble pairs (10–12). Multiple types of G•A mismatch pairs with two hydrogen bonds have been discovered (13–16). These include the *cis* Watson–Crick/Watson–Crick (*cis* W.C.), the *cis* Watson–Crick/Hoogsteen, the *cis* Sugar-edge/Sugar-edge, the *trans* Hoogsteen/Sugar-edge (`sheared’ G•A), and the *trans* Sugar-edge/Sugar-edge. Although less common than the sheared G•A, the *cis* W.C. G•A pair plays important structure/function roles in various natural RNAs (15,17–21). For example, the *cis* W.C. G•A pair is essential for the catalysis observed in the autocatalytic genomic hepatitis delta virus (HDV) ribozyme. The deletion of the adenine results in the decreased activity of the genomic ribozyme (15,19). The crystal structure of the P4–P6 domain of the *Tetrahymena* group I intron contains two *cis* W.C. G•A pairs in separate locations, which occur at the interface between helices and other motifs.
strongly suggesting a possible functional role of these cis W.C. G•A pairs in stabilizing RNA folding (19). Many examples of cis W.C. G•A pairs were found in the crystal structure of the 30S ribosomal subunit (16S rRNA) of H.thermophilus and the 50S ribosomal subunits (23S rRNA) of the archaean H.marismortui and the bacterium D.radiodurans (1,15,17,18). The highly conserved cis W.C. G•A base pairs seen in these structures are mostly involved in either tertiary or neighbor contacts in the ribosome (15). Although the majority of the known G•A mismatch pairs occur as singlets, two or more consecutive GA oppositions such as those seen in helix 42 do occur and multiple examples are found in the ribosomal structures. Many of these are in bulge loops or junction regions rather than being embedded in stem structures. In 18 of the 23 known ribosomal examples (1JJ2.pdb, 1J5E.pdb), both neighboring GA oppositions adopt the sheared base pair conformation and in most cases the adenines are on opposite strands. One of a few examples with the adenines on the same strand involves positions 5′ G816G817 3′/3′ A796A797 5′ in the H.marismortui 50S ribosomal subunit. In the remaining five examples, one of the G•A oppositions forms either one or no hydrogen bond while the other is a sheared G•A pair. Tandem sheared (G•A)₂ oppositions have also been seen in the group I intron ribozyme (22,23) where they are associated with a change in direction of the backbone that allows creation of a three-helix junction. In contrast, no instances of the cis W.C. G•A oppositions were observed in the helix stem of 23S rRNA (1S72.pdb). Instead of a base pair next to the G•A oppositions were now of the cis W.C. type (27) in the analog but were the more usual sheared configuration in the larger context of the intron. This was not unexpected as the structural context in the intron was dramatically different from that of the analog (23). Instead of a base pair next to the G•A oppositions, the intron helix immediately opens up to form a junction. In total, these results suggested that the G•A oppositions in helix 42 would also be of the cis W.C. type, but given the well-established context sensitivity of such interactions this was by no means certain. Metal ions have long been known to stabilize RNA structure (28) and have been implicated in stabilizing the catalytic core of numerous ribozymes (29). A recent study (30) greatly extended our knowledge in this area by examining the major role played by Mg²⁺ ions in maintaining the structural integrity of the H.marismortui 23S rRNA. Four Mg²⁺ ion binding sites were observed in the helix stem of 23S rRNA (1S72.pdb). The metal ion binding was seen to be especially abundant in and around the peptidyl transferase center, where ribosomal proteins are absent, reminding us that metal ions may have been of special significance in a primordial RNA world. In addition to the examples in the ribosome structure, a tandem (G•A)₂ in a P5GA hairpin of a group I intron has been observed to form a Mg²⁺ ion binding site (23,27). In the work described herein, we have used NMR spectroscopy to make a detailed examination of the structure of a base was a G, the structure was dramatically changed with the result that the G•A pairs were now of the cis W.C. type resulting in a widening of the backbone (26). The one study to date in which the adenines in the G•A oppositions were on the same strand was a small stem which was in part analogous to the P5abc domain of the group I intron (27) The sequence context in this case differs from that seen in E.coli helix 42 in that the 3′ pair is a wobble pair rather than a standard Watson–Crick pair. In this case, the G•A oppositions were of the cis W.C. type (27) in the analog but were the more usual sheared configuration in the larger context of the intron. This was not unexpected as the structural context in the intron was dramatically different from that of the analog (23). Instead of a base pair next to the G•A oppositions, the intron helix immediately opens up to form a junction. In total, these results suggested that the G•A oppositions in helix 42 would also be of the cis W.C. type, but given the well-established context sensitivity of such interactions this was by no means certain. Metal ions have long been known to stabilize RNA structure (28) and have been implicated in stabilizing the catalytic core of numerous ribozymes (29). A recent study (30) greatly extended our knowledge in this area by examining the major role played by Mg²⁺ ions in maintaining the structural integrity of the H.marismortui 23S rRNA. Four Mg²⁺ ion binding sites were observed in the helix stem of 23S rRNA (1S72.pdb). The metal ion binding was seen to be especially abundant in and around the peptidyl transferase center, where ribosomal proteins are absent, reminding us that metal ions may have been of special significance in a primordial RNA world. In addition to the examples in the ribosome structure, a tandem (G•A)₂ in a P5GA hairpin of a group I intron has been observed to form a Mg²⁺ ion binding site (23,27). In the work described herein, we have used NMR spectroscopy to make a detailed examination of the structure of a
MATERIALS AND METHODS

RNA sample preparation

The 24mer RNA oligonucleotide 5’-GGGAAGGCCUCG-GCGUCGGCCC-3’ was synthesized by in vitro transcription using T7 RNA polymerase and a synthetic DNA template. Unlabeled RNA samples were prepared from 20 ml transcription reactions using 4 mM 5’-NTPs as described elsewhere (33–36). Aliquots (1 mM) of 13C- and 15N-labeled ATP and GTP (Silantes) were used to uniformly label the RNA samples in 20 ml transcription reactions (37). The RNA samples were purified by electrophoresis on 20% polyacrylamide denaturing gels and eluted with RNA elution buffer (0.5 M NH4OAC and 1 mM EDTA, pH 7.0). The samples were desalted with a Sep-pak C-18 cartridge (Waters Inc.) and dialyzed against an NMR buffer containing 10 mM NaH2PO4/Na2HPO4, 0.1 mM EDTA, pH 6.5 using an Amicon-3 concentrator (Millipore) (38). For exchangeable proton measurements, 8% D2O was used to provide the lock signal. For non-exchangeable proton measurements, the samples were lyophilized three times and then redissolved in 99.96% D2O. The unlabeled sample was then dissolved in 0.50 ml of either 92% H2O:8% D2O or 99.96% D2O (Cambridge Isotope Laboratories, Inc.) to a final concentration of 1.4 mM. The uniformly labeled sample was dissolved in 0.25 ml in a 5-mm Shigemi tube with a final concentration of 1.4 mM.

NMR spectroscopy

NMR spectra were acquired on a Bruker Avance 600 MHz NMR spectrometer. All NMR data were processed and analyzed with the programs XWINNMR (Bruker Instruments) and Sparky (39). One-dimensional (1D) melting profiles and 2D NOESY spectra in 92% H2O/8% D2O were collected to study the exchangeable protons in the temperature range 0–25°C using jump-return pulses (40) to suppress the water peak. The samples were analyzed in the 99.96% D2O to observe non-exchangeable protons. The 2D NOESY spectra (50, 100, 250 and 500 ms mixing times), TOCSY with 80 ms mixing time were acquired in the temperature range 12–35°C. DQF-COSY spectra were recorded at 25 and 35°C using a repetition delay of 4.2 s. A proton-detected heteronuclear 31P–1H COSY spectrum was acquired with sweep width 8389.3 Hz in the 1H dimension and 458.8 Hz in the 31P dimension at 25°C.

Experiments on uniformly labeled RNA samples were performed in 92% H2O/8% D2O at both 12°C and 25°C. 13C–1H HSQC spectra were acquired with the 13C carrier at 143.0 p.p.m., the spectral width in the 13C dimension of 3773 Hz and the spectral width in the 1H dimension of 8389 Hz. 15N–1H HSQC spectra were acquired with the 15N carrier at 147.0 p.p.m. The spectral width was set to 912 Hz in the 15N dimension and 12019 Hz in the 1H dimension. The quantitative JNN HNN-COSY experiments were acquired with a total of 128 scans per FID. The spectral width was 6081 Hz in the 15N dimension and 12019 Hz in the 1H dimension. The experiments were performed with a 1H carrier positioned on the H2O resonance, the 15N carrier at 185.0 p.p.m. and 1H carrier at 153.0 p.p.m. A mixing time of 1 s was applied for HNN-COSY transfer.

The effect of Mg2+ on the 24mer RNA fragment was monitored by chemical shift measurements from the 1H–15N HSQC spectra of the uniformly labeled RNA in 92% H2O/8% D2O and the 1D NOESY spectra of the non-labeled sample in 99.96% D2O upon the addition of 0, 5, 10, 15, 20, 25, 30, 35 and 40 mM Mg2+. In order to avoid sample dilution during the Mg2+ titration, 5 mM MgCl2 aliquots were dried and then the RNA samples were sequentially combined with each of the nine aliquots to increase the Mg2+ concentration from 0 to 40 mM, while keeping the sample volume at 0.5 ml (unlabeled sample) or 0.25 ml (labeled sample). The pH of the sample was 6.5 during the Mg2+ titration. 15N T1 and T2 and 1H–15N NOE spectra were acquired at 25°C with the pulse sequences as described previously (41,42). 15N T1 values were measured from the spectra recorded with seven different relaxation delays: 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 s. T2 values were calculated from spectra recorded with seven different delays: 0.016, 0.047, 0.079, 0.110, 0.142, 0.173, 0.205 s. 1H–15N NOE experiments were performed with the repetition delay of 4.0 s. A repetition delay of 2.0 s was employed in the measurement of 15N T1 and T2 values. The standard deviation values of the order parameters and phenom- enological exchange term were indicated by the sim error (43).

NMR constraints and structure calculations

Structure calculations utilized procedures described previously (38). The cross-peaks were assigned for all the 2D NOESY spectra in D2O (50 ms, 250 ms and 500 ms mixing times) and H2O (200 ms mixing time) (Supplementary Material Table S1). Distance restraints were derived from the cross-peak intensities by categorizing the NOEs as strong (1.8–3.2 Hz), medium (3.6–5.0 Hz) or weak (5.6–7.0 Hz) corresponding to C2-end conformation. Other residues were in the range of a C3-end conformation. Other residues were in the range of a C3–endo conformation corresponding to a δ torsion angle of 140° ± 20° corresponding to C2-end conformation. Other residues were in the range of a C3–endo conformation corresponding to a δ torsion angle of 80° ± 20°. Chi (–160° ± 20°) is correlated with C3–endo and (–130° ± 15°) with C2–endo. The residue G15 with a downfield phosphorus resonance was
restrained to the torsion angles as reported previously (44–46). With the exception of the tandem (G\bullet A)₂, the backbone torsion angles of the other stem residues were loosely constrained to the range of A-form parameters (α = −68° ± 15°, β = 178° ± 15°, γ = 54° ± 15°, ε = −153° ± 15° and ζ = −71° ± 15°) (38).

The restrained molecular dynamics (rMD) simulated annealing protocol in the Crystallography and NMR System (CNS) package was used to determine the structure (47). A total of 550 distance restraints, 120 dihedral restraints (Table 1) and 11 planarity restraints were incorporated into rMD followed by energy minimization with an all-atom force field (47). The initial structures were generated in extended strands in random dihedral angles. After global folding and refinement, the converged structures were accepted on the basis of low total energy, the avoidance of bond violation >0.10 Å, torsion angle violation >5.0°, improper violation >15.0°, dihedral violation >20° (38), vdw violation >1.7 and NOE distance violations >0.3 Å. Structures were viewed with VMD (48) and analyzed with X3DNA (49).

### Backbone dynamics analysis

R₁ and R₂ values of ¹⁵N-labeled G residues were determined by fitting exponential peak height decay in a series of ¹⁵N T₁ and T₂ spectra respectively using the program Sparky (39). The average values of T₁ and T₂ are 0.47 and 0.14 s, respectively. The relaxation parameters of ¹⁵N of the G residues were derived. The values of R₁, R₂, NOE, S², and R_ex are shown in Supplementary Material Table S2. The phenomenological exchange term R_ex indicated the contribution of chemical exchange processes to the decay of transverse magnetization through the relaxation measurements. The extent of picosecond-time scale motions were determined by the order parameter S². The generalized order parameters S² and phenomenological exchange term R_ex were calculated with Modelfree 4.0 (43) using the Lipari–Szabo approach (50,51). A grid search of internal motional parameters was performed before optimization of the overall diffusion model. Brent’s implementation of the Powell’s method was applied to the optimization of the axially symmetric diffusion tensor for all spins (43). A simple spectral density function including S² and R_ex (52) was selected to fit the experimental NMR transverse relaxation data for the extraction of the motional parameters. The average value of the order parameter S², over all residues except those in the tandem (G\bullet A)₂ region, is 0.78. The order parameters S² of G20 and G21 are 0.78 and 0.91, respectively. In contrast to the neighboring residues, residue G21 exhibits higher R_ex contribution to the transverse relaxation rate, arising from conformational processes, of 2.58 ± 0.643 s⁻¹.

### RESULTS

#### Spectral analysis

Assignment of exchangeable protons. 2D NOESY spectra at 5°C in 92% H₂O/8% D₂O are used to assign exchangeable protons. Eleven imino proton resonances are shown in the 1D proton spectrum of the 24mer RNA sample. The starting point for imino proton assignments was established by identification of the G7\bullet U18 wobble pair, which provides a very strong cross-peak between the G7 imino and U18 imino protons in the 2D NOESY spectra. The sequential walk was evident based on the characteristic downfield imino resonance of U18 and the upfield shifted G7 imino. G6 was identified by the cross-peak between G6 and G7 imino protons. G17 was determined by the cross-peak between G17 and U18. The identification of the imino protons of G9, G2 and G3 was facilitated by the observation of cross-peaks between G(H1)–C(NH2) and C(NH2)–C(H5). This is the characteristic pattern associated with Watson–Crick G–C base pairs.

Among the remaining imino peaks, two resonated in the region between 11.0 and 12.0 p.p.m. This is the typical chemical shift range for the guanine imino protons that are found in cis W.C. G\bullet A mismatches (14). The imino peak at 11.2 p.p.m. had a cross-peak to G3 imino proton and therefore was assigned to G21. The other non-canonical base-pairing pattern was assigned to residue G20. The intense cross-peaks between A5 H2 and the G20 amino group are assigned unambiguously. Cross-peaks between A4 H2 and the G21 amino group were also observed. This indicates that both G\bullet A base pairs are of the cis W.C. G\bullet A type.

Assignment of non-exchangeable protons. The 2D-NOESY and DQF-COSY spectral analysis confirmed formation of a hairpin by the 24mer RNA. Supplementary Material Figure S1 shows the expanded base (H8/H6/H2) to sugar (H1’/H5) region of the NOESY spectrum recorded with 500 ms mixing time at 25°C. All the signature spectral features of the well-characterized UUCG tetraloop (44–46) were observed in the 2D spectra. The sequential connectivities are absent at the loop residues U11, U12, G14 and G15. In particular, G14 adopts a syn conformation with the very strong H1’–H2 and intense intranucleotide H8–H1’ cross-peaks even in the 50 ms 2D NOESY spectra. The H1’ of the adjacent G15 is unusually upfield shifted (44–46). The sequential NOEs of residues 2 through 10 and 15 through 23 are consistent with the duplex stem formation, except that the internucleotide cross-peaks of A4H1’/A5H8 and G21H1’/C22H6 are absent. This indicates

---

Table 1. Statistics of NMR restraints (47) in the structure calculation and RMSD values among the final converged structures

| (1) Distance restraints | Number of violations |
|------------------------|---------------------|
| Intra-residue          | 316                 |
| Inter-residue          | 211                 |
| Watson–Crick           | 23                  |
| Mean number per residue| 23                  |

| (2) Dihedral restraints | Total NMR derived number | Mean number per residue |
|------------------------|--------------------------|-------------------------|
|                        | 120                      | 5                       |

| (1) Violations | Number of violations |
|---------------|----------------------|
| Bond > 0.10 Å| 0                    |
| Angle > 5.0° | 0                    |
| Improper > 15.0° | 0             |
| Dihedral > 20.0° | 0              |
| Vdw > 1.7  | 0                    |
| NOE > 0.3 Å | 0                    |

| (2) RMSD of residues (Å) | 0.85 ± 0.50 |
|--------------------------|-------------|
| 9 structures (all nt)    | 0.77 ± 0.38 |
| 9 structures (stem)      | 0.72 ± 0.09 |
| 9 structures (loop)      | 0.29 ± 0.09 |

---

3148 Nucleic Acids Research, 2005, Vol. 33, No. 10

Downloaded from https://academic.oup.com/nar/article-abstract/33/10/3145/1009083 by guest on 29 July 2018
local structural alteration at the tandem (G•A)₂ site (27). However, the presence of coupling cross-peaks interstrand G(N1)–G(H1)–A(N1) correlations in HNN-COSY spectrum (Figure 2) unambiguously reveals the form of cis W.C. G•A pairs.

**Assignments of ³¹P resonances.** Nineteen chemical shifts of ³¹P were assigned with chemical shift dispersing over a range of 2.57 p.p.m. [from 1.76 (G15) to −0.81 p.p.m. (C13)]. Most large deviations of the ³¹P chemical shifts are from the loop residues or those at the junction of the loop to stem; the ³¹P resonances of G3–A5 and of C19–G21 appeared at a narrow chemical shift range (0.8 p.p.m.), indicating the tandem (G•A)₂ adopts a regular helical backbone conformation.

**Backbone dynamics.** Backbone dynamics measurements and modeling results (Supplementary Material Figure S3 and Table S2) did not reveal any clear abnormalities in relaxation, the order parameters or the phenomenological exchange term. This indicates that the tandem (G•A)₂ does not induce additional amplitude motions at a picosecond to nanosecond time scale. The small variations observed in ⁵⁷² for the ⁵⁷-terminal residues G2 and G3 do not provide sufficient information for isolation of local dynamics.

**Specific spectral features of the tandem (G•A)₂**

The tandem (G•A)₂ exhibits well-defined spectral patterns. The imino protons of G20 and G21 were clearly observed and they can be linked to A(N1) through the measurement of JNN in the heteronuclear correlation spectrum. The amino protons of both G residues are in close contact with the corresponding H2 of the A residues. These spectral observations provide evidence for a well-defined cis W.C. type of tandem (G•A)₂ with the two G and A residues in opposite strands (Figure 3a). Stacking between the two G•A pairs was observed from the adjacent imino protons of G residues to the adjacent H8 protons of G and A residues, establishing that the cis W.C. type of tandem (G•A)₂ pairs are well-accommodated in the hairpin stem helix.

**Overall structure**

An overlay of the nine alternative structures generated is shown in Figure 3b and the structural calculation statistics are provided in Table 1. The global structure of the converged hairpin stem generally resembles the standard A-form geometry. The UUCG loop is very similar to that described previously (44–46), with the U11•G14 pair stabilized by base–base and base–sugar hydrogen bonds and the syn conformation of G14 as well as C2′-endo sugar puckering of U12 and C13. The unusually upfield shifted H1' resonance of residue G15 is attributed to the ring current effects of the syn base of residue G14 (44). The overall average helical twist of the stem region is 29.9° which is close to the 32.7° (24) associated with the standard A-form (Supplementary Material Table S3). The stem region contains a G•U wobble pair, which exhibits unwinding at the step 5' to G but overwinding at the step 3' to G (Supplementary Material Table S3, 20.5° versus 42.2°). The tandem (G•A)₂ region deviates from the average helical twist in part for the reduced overall average. Specifically, the helical twists of A4G21–A5G20, A5G20–G6C19 are 25.3° and 28.1°, respectively, suggesting an underwinding between these cis W.C. G•A mismatches compared to the A-form helix (24). This underwinding of the helix at the mismatch is associated with the intrastrand stacking of the cis W.C. G•A mismatches. In contrast, G3C22–A4G21 is clearly overwound with a twist 38.8°. The twist angle of G6C19–G7U18 is 20.5° which is close to the 32.7° (24) associated with the standard A-form geometry. The tandem (G•A)₂ region deviates from the average helical twist in part for the reduced overall average. Specifically, the helical twists of A4G21–A5G20, A5G20–G6C19 are 25.3° and 28.1°, respectively, suggesting an underwinding between these cis W.C. G•A mismatches compared to the A-form helix (24). This underwinding of the helix at the mismatch is associated with the intrastrand stacking of the cis W.C. G•A mismatches. In contrast, G3C22–A4G21 is clearly overwound with a twist 38.8°. The twist angle of G6C19–G7U18 is 20.5° which is close to the 32.7° (24) associated with the standard A-form geometry. The tandem (G•A)₂ exhibits well-defined spectral patterns. The imino protons of G20 and G21 were clearly observed and they can be linked to A(N1) through the measurement of JNN in the heteronuclear correlation spectrum. The amino protons of both G residues are in close contact with the corresponding H2 of the A residues. These spectral observations provide evidence for a well-defined cis W.C. type of tandem (G•A)₂ with the two G and A residues in opposite strands (Figure 3a). Stacking between the two G•A pairs was observed from the adjacent imino protons of G residues to the adjacent H8 protons of G and A residues, establishing that the cis W.C. type of tandem (G•A)₂ pairs are well-accommodated in the hairpin stem helix.

**Mg²⁺ binding in the tandem (G•A)₂**

Chemical shifts are sensitive to metal ion binding and its induced structural changes. The potential binding of Mg²⁺ to the 24mer RNA hairpin was investigated by monitoring the ¹H and ¹⁵N chemical shifts of the G and A residues. The induced changes in the chemical shifts of the imino-N1, imino-H of guanines and H8/H6 protons of all the residues were monitored and plotted as a function of increasing concentration of Mg²⁺ up to 40 mM (Supplementary Material Figure S2). The observed patterns of chemical shift variation...
for the imino nitrogens and protons are summarized in Figure 4. The majority of the net chemical shift changes, though subtle, are shown in clearly defined trends (Supplementary Material Figure S2). While the resonances of G20 are associated with the largest (Figure 4, marked with ‘1’) upfield shift with increasing Mg\(^{2+}\) concentration, other residues around the tandem (GA)\(_2\), i.e. the chemical shifts of G3NH, G20NH, G20N1, G20H8 and A4H8, were also affected by the addition of Mg\(^{2+}\) (Figure 4). These resonance perturbations relate to major groove functional groups of residues central to the hairpin stem, suggesting the localization of the Mg\(^{2+}\) ion in the major groove and at the vicinity of the tandem (G•A)\(_2\) pair (27). Mg\(^{2+}\) binding is commonly found near the phosphate groups of ribosomal RNA (30), and when it occurs the interaction tends to be non-specific and mediates RNA–protein complex formation. Our results do not exclude this binding mode of Mg\(^{2+}\) in the 24mer RNA.

We further examined possible modes of Mg\(^{2+}\) binding by docking the metal ion at the cis W.C. type of tandem (G•A)\(_2\) site (Figures 5a and 6b). The alignment of the G and A bases...
with their Watson–Crick edge forming hydrogen bonds presents juxtaposed electron donors: O6 from G20 and N1 from A4. The distance (2.3 Å) between these ligands and the Mg²⁺ suggests that they are interacting with the inner-sphere (30). The close contacts of these atoms with the metal ion would be expected to introduce electron inductive and conjugation effects in the nucleobase ring system, giving rise to the observed chemical shift changes, while the resonances in other regions of the 24mer RNA hairpin are essentially insensitive to the addition of Mg²⁺. The divalent metal ion binding bridging the cross-strand G and A residues would require two consecutive base pairs. The geometry of the tandem (G•A)₂ region determined here is consistent with the proposed Mg²⁺ binding site. The interphosphate distance of the tandem (G•A)₂ region (between 18 and 20 Å) is more open than the normal distance of an A-form helix. This wider major groove allows the tandem (G•A)₂ region to present a larger surface for Mg²⁺ binding and potentially for auxiliary water to participate as an outer-sphere ligand in stabilizing the metal ion coordination. The major groove of the tandem G•A sequences is shown to be electron negative (30), offering additional attractive force for the binding of positively charged ions. The tandem (G•A)₂ region described in this work provides an additional example of Mg²⁺ binding in an A-form helix.

Comparison of the NMR structure with 23S rRNA crystal structures

From a comparative biology perspective, the E.coli version of helix 42 is expected to closely resemble the equivalent regions in other 23S rRNAs despite the presence of the tandem (G•A)₂. In order to examine this, the NMR structure determined here and the corresponding regions in the crystal structures of the H.marismortui (1JJ2.pdb) and D.radiodurans (1NKW.pdb) 50S ribosomal subunits were superimposed. The NMR structure has an RMSD value of 2.64 Å with H.marismortui and 2.80 Å with D.radiodurans, whereas the two crystal structures differ with an RMSD value of 1.67 Å. Figure 3c compares the overall geometry of the NMR structure with the equivalent region in H.marismortui. The typical structural features of RNA, a very deep major groove and a rather shallow minor groove are clearly observed in the corresponding regions in both structures. The NMR structure...
has clearly adapted what is essentially an A-form geometry despite the presence of the tandem (G●A)₂ as confirmed by examination of the helical parameters (Supplementary Material Tables S3 and S4). The geometry of the G●A mismatches determined by NMR is also consistent with the primary sequence exchanges between GA and W.C. oppositions that are seen in the primary sequences of the E.coli and H.marismortui 23S RNAs.

**DISCUSSION**

It has been unequivocally shown that the two G●A oppositions in the analog of the lower portion of helix 42 in E.coli 23S rRNA form pairs of the cis W.C. type in solution. In view of the comparative sequence data and the number of canonical Watson–Crick base-pairs flanking the two G●A oppositions, it is virtually certain that the structure seen here will be essentially the same as that is found in the 23S rRNA. Thus, the lower portion of helix 42 in E.coli 23S rRNA represents the first known occurrence of tandem (G●A)₂ of the cis W.C. type embedded in an otherwise normal stem structure in a naturally occurring functional RNA.

The cis W.C. G●A mismatch is frequently found at the ends of canonical helices where it provides an interface between the helix and other structural motifs (15). However, in the present case it is located within a canonical helix accompanied by an equally unusual Mg²⁺ binding site. The cis W.C. geometry of the tandem (G●A)₂ allows N1 of A4 to coordinate with the Mg²⁺ while the Mg²⁺ serves as a bridge between G20 and A4 via O6 of G20 on the other side. This Mg²⁺ binding duplex motif allows the Mg²⁺ to locate in the major groove of the helix.

The vast majority of Mg²⁺ binding sites in the best-studied case, H.marismortui 23S rRNA, occur at helical bulges and interhelical junctions. For example, there is one Mg²⁺-binding site at an interhelical junction in H.marismortui 23S rRNA that consists of non-canonical tandem (G●A)₂ with G residues on same side. The two O6 atoms serve as inner-sphere coordination sites for the Mg²⁺ (Figure 6a). On the opposite side, neither A796 nor A797 is involved because the sheared conformation of the tandem (G●A)₂ shields them from the Mg²⁺ site. Thus, despite the sequence similarity the Mg²⁺ coordination in this case is quite different than seen in the structure studied here.

Only 4 of 116 Mg²⁺ ions were found to bind to residues in helical stem regions in the large ribosomal subunit of H.marismortui (30). In just one case (Figure 6b), the Mg²⁺ has a bridge role similar to that seen in the helix 33 analog. In this instance, G863–C783 and G782 has a bridge role similar to that seen in the helix 33 analog. The two O6 atoms serve as inner-sphere coordination sites for the Mg²⁺ and Mg²⁺ bridging the cross-stand residues G863 and G782. The inner-sphere ligand for Mg²⁺ is from O6 atom of G863 and O4 atom of U864 as well (figure not shown). O6 atom of G782 provides outer-sphere ligand for Mg²⁺ while N7 of G863 is simultaneously coordinated to the Mg²⁺ as another outer-sphere ligand. The result is a structure with isoteric similarity to the Mg²⁺ binding site proposed here for the E.coli helix 42 analog. This suggests that the cross-strand bridge may be a general but rare motif in Mg²⁺ coordination.

The rare occurrence of the 5’ GG/3’ AA (cis W.C.) tandem interaction and its accompanying Mg²⁺ coordination suggests that the structure obtained here may be uniquely suitable for inclusion in helix 42 of 23S rRNA. The obvious question is ‘what functional significance if any this unusual stem structure has?’ The structural comparisons (Figure 3c) show that the inclusion of the two imino pairs minimally disrupts the overall structure. The backbone dynamics reveal that residue G21 (E.coli position 1116) has far more potential for slow conformational change than the surrounding residues. Thus, one possibility is that the non-canonical pairs may facilitate flexibility in this stem. Consistent with this, the equivalent positions in H.marismortui (but not D.radiodurans) are relatively weak pairs. Metal ion binding sites are frequently found to be important in stabilizing RNA structures required for specific protein recognition in the large ribosomal subunit (30). However, if the H.marismortui 50S subunit crystal structure is representative, the lower portion of helix 42 may not be in close proximity to any proteins. It is not unreasonable to speculate that as a result of one or more of the conformational changes known to occur in the L7/L12 stalk, this situation changes. Thus, the importance of the tandem (G●A)₂ may be that it provides flexibility which is needed to accommodate the functionally important conformational changes that occur in the L7/L12 stalk. It must, nevertheless, be emphasized that this analog structure was investigated in the absence of the ribosomal proteins and therefore may lack subtle structural rearrangements upon binding of nearby ribosomal proteins.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at NAR Online.

**ACKNOWLEDGEMENTS**

We thank the W.M. Keck Foundation which partially funds the 600 MHz NMR spectrometer at the University of Houston. The Institute of Molecular Design and the W.M. Keck Center provided computer resource support. The work was supported by grants from the Robert A. Welch Foundation (E-1451) and the National Aeronautics and Space Administration (NAG5-12366) to G.E.F, and the Robert A. Welch Foundation (E-1270) and NIH/GM (RO149957) to X.G. Funding to pay the Open Access publication charges for this article was provided by indirect cost funds.

Conflict of interest statement. None declared.

**REFERENCES**

1. Ban,N., Nissen,P., Hansen,J., Moore,P.B. and Steitz,T.A. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science*, 289, 905–920.
2. Ostergaard,P., Phan,H., Johansen,L.B., Egeberg,J., Ostergaard,L., Porse,B.T. and Garrett,R.A. (1998) Assembly of proteins and 5S rRNA to transcripts of the major structural domains of 23 S rRNA. *J. Mol. Biol.*, 284, 227–240.
3. Klein,D.J., Schmeing,T.M., Moore,P.B. and Steitz,T.A. (2001) The kink-turn: a new RNA secondary structure motif. *EMBO J.*, 20, 4214–4221.
4. Baranov,P.V., Gurvich,O.I., Bogdanov,A.A., Brimacombe,R. and Dontsova,O.A. (1998) New features of 23S ribosomal RNA folding: the long helix 41–42 makes a “U-turn” inside the ribosome. *RNA*, 4, 658–668.
5. Mohr, D., Winternayer, W. and Rodríguez, M.V. (2002) GTPase activation of elongation factors Tu and G on the ribosome. *Biochemistry*, 41, 12520–12528.

6. Lu, M. and Draper, D.E. (1995) On the role of RNA tertiary structure in recognition of ribosomal protein L11 and thiostrepton. *Nucleic Acids Res.*, 23, 3426–3433.

7. Gonzalo, P. and Reboud, J.P. (2003) The puzzling lateral flexible stalk of the ribosome. *Biol. Cell*, 95, 179–193.

8. Zhao, Q., Oeverstedt, L.G., Skoglund, U. and Isaksson, L.A. (2004) Morphological variation of individual *Escherichia coli* 30S ribosomal subunits in situ, as revealed by cryo-electron tomography. *Exp. Cell Res.*, 300, 190–201.

9. Christodoulou, J., Larsson, F., Fucini, P., Connell, S.R., Pertinhez, T.A., Hanson, C.L., Redfield, C., Nierhaus, K.H., Robinson, C.V., Schleucher, J. et al. (2004) Heteronuclear NMR investigations of dynamic regions of intact *Escherichia coli* ribosomes. *Proc. Natl Acad. Sci. USA*, 101, 10949–10954.

10. Noller, H.F. (1984) Structure of ribosomal RNA. *Annu. Rev. Biochem.*, 53, 119–162.

11. Cech, T.R. and Bass, B.L. (1986) Biological catalysis by RNA.

12. Cech, T.R. and Doudna, J.A. (1996) Crystal structure of a group I ribozyme from *H. marismortui*. Nature *380*, 327–339.

13. Clore, G.M., Driscoll, P.C., Wang, J. and Sykes, B.D. (1989) Analysis of the backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by 15N NMR relaxation. *Biochemistry*, 33, 5984–6003.

14. Clore, G.M., Driscoll, P.C., Wingfield, P.T. and Gronenborn, A.M. (2006) Analysis of the backbone dynamics of interleukin-1 beta using two-dimensional inverse detected heteronuclear 15N-1H NMR spectroscopy. *Proteins Struct. Funct. Genet.*, 64, 1366–1379.

15. Colmenarejo, G. and Tinoco, I.Jr (1999) Structure and thermodynamics of the human immunodeficiency virus reverse transcriptase using 15N relaxation measurements. *Biochemistry*, 38, 5129–5139.

16. Leontis, N.B., Stombaugh, J. and Westhof, E. (2002) The non-Watson–Crick A/G base pairs and their associated isostericity matrices. *Nucleic Acids Res.*, 30, 3497–3531.

17. Wu, M. and Turner, D.H. (1996) Solution structure of (rGCGGACGC)2 tandem GA mismatches in RNA depend on closing base pairs. *Biochemistry*, 35, 967–978.

18. Harms, J., Schluenzen, F., Zarivach, R., Bashan, A., Agmon, I., Gish, G., Shoelsom, S.E., Watson, P., Forman-Kay, J.D. and Kay, L.E. (1994) Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by 15N NMR relaxation. *Biochemistry*, 33, 5984–6003.

19. Liley, D.M. (2003) The origins of RNA catalysis in ribozymes. *Trends Biochem. Sci.*, 28, 495–501.

20. Klein, D.J., Moore, P.B. and Steitz, T.A. (2004) The contribution of metal ions to the structural stability of the large ribosomal subunit. RNA, 10, 1366–1379.

21. Harms, J., Schluenzen, F., Zarivach, R., Bashan, A., Agmon, I., Gish, G., Shoelsom, S.E., Watson, P., Forman-Kay, J.D. and Kay, L.E. (1994) Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by 15N NMR relaxation. *Biochemistry*, 33, 5984–6003.

22. Clore, G.M. (1998) Structure and thermodynamics of metal binding in the P1 helix from group I self-splicing introns. *J. Mol. Biol.*, 280, 333–353.

23. Colmenarejo, G. and Tinoco, I.Jr (1999) Structure and thermodynamics of metal binding in the P5 helix of a group I intron ribozyme. *J. Mol. Biol.*, 290, 119–135.

24. Wong, K.H., DeLange, T.W. and Gros, P. (1996) Analysis of the backbone dynamics of interleukin-1 beta using two-dimensional inverse detected heteronuclear 15N-1H NMR spectroscopy. *Biochemistry*, 35, 967–978.

25. Humphrey, W., Dalke, A. and Schulten, K. (1996) VMD: visual molecular dynamics. *J. Mol. Graph.*, 14, 33–38, 27–38.

26. Lu, X.J. and Olson, W.K. (2003) 3DNA: a software package for the analysis, rebuilding and visualization of three-dimensional nucleic acid structures. *Nucleic Acids Res.*, 31, 5108–5121.

27. Rudisler, S. and Tinoco, I.Jr (2000) Solution structure of Cobalt(III)hexamine complexed to the GAA tetraloop, and metal-ion binding to G.A mismatches. *J. Mol. Biol.*, 295, 1211–1223.

28. Draper, D.E. (2004) A guide to ions and RNA structure. RNA, 10, 335–343.