Structure of 23S rRNA hairpin 35 and its interaction with the tylosin-resistance methyltransferase RlmA^{II}

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The bacterial rRNA methyltransferase RlmA^{II} (formerly TlrB) contributes to resistance against tylosin-like 16-membered ring macrolide antibiotics. RlmA^{II} was originally discovered in the tylosin-producer *Streptomyces fradiae*, and members of this subclass of methyltransferases have subsequently been found in other Gram-positive bacteria, including *Streptococcus pneumoniae*. In all cases, RlmA^{II} methylates 23S rRNA at nucleotide G748, which is situated in a stem-loop (hairpin 35) at the macrolide binding site of the ribosome. The conformation of hairpin 35 recognized by RlmA^{II} is shown here by NMR spectroscopy to resemble the anticodon loop of tRNA. The loop folds independently of the rest of the 23S rRNA, and is stabilized by a non-canonical G–A pair and a U-turn motif, rendering G748 accessible. Binding of *S. pneumoniae* RlmA^{II} induces changes in NMR signals at specific nucleotides that are involved in the methyltransferase–RNA interaction. The conformation of hairpin 35 that interacts with RlmA^{II} is radically different from the structure this hairpin adopts within the 50S subunit. This indicates that the hairpin undergoes major structural rearrangement upon interaction with ribosomal proteins during 50S assembly.

Keywords: NMR spectroscopy/macrolide/protein–RNA interaction/rRNA methylation/translation

Introduction

RlmA^{II} (formerly TlrB) is a subclass of ribosomal RNA (rRNA) methyltransferases that are associated with resistance to tylosin and structurally similar 16-membered ring macrolide antibiotics (Liu and Douthwaite, 2002a). RlmA^{II} catalyzes the transfer of a single methyl group from *S*-adenosylmethionine to the base of G748 within hairpin 35 of bacterial 23S rRNA (Liu *et al.*, 2000). Nucleotide G748 is located in the peptide exit channel of the 50S ribosomal subunit at the site where macrolide, lincosamide and streptogramin B (MLS_B) antibiotics bind, and is ~30 Å from the peptidyl transferase centre (Ban *et al.*, 2000; Schlünzen *et al.*, 2001;

Yusupov *et al.*, 2001). RlmA^{II} is homologous to the methyltransferase RlmA^I (formerly RrmA), which is specific for nucleotide G745, and is found in many Gram-negative bacteria including *Escherichia coli* (Gustafsson and Persson, 1998; Liu and Douthwaite, 2002b). With the exception of their *S*-adenosylmethionine binding motifs, RlmA^I and RlmA^{II} show little sequence similarity to the well-characterized Erm methyltransferases, which methylate 23S rRNA nucleotide A2058 and thereby confer resistance to all MLS_B drugs. When 23S rRNA is assembled within the 50S subunit, nucleotides G745, G748 and A2058 come to lie within 15 Å of each other, and their methyl groups point into the lumen of the peptide exit channel. Within the 50S subunit these positions are no longer accessible to methyltransferases, and consistent with this RlmA^I, RlmA^{II} and Erm methylate their target nucleotides only in the free rRNA prior to assembly with the ribosomal (r) proteins.

The interaction of the *E. coli* RlmA^I with its target in 23S rRNA has recently been investigated by chemical footprinting, and the rRNA contacts were shown to be confined to hairpin 35, the two adjacent helices 33 and 34, and the three-way junction linking these helices (Figure 1) (Hansen *et al.*, 2001). Small RNA transcripts consisting of these three helices also function as efficient substrates for RlmA^I, indicating that all the elements required for recognition by the enzyme are contained within these rRNA structures. The extensive sequence similarity between RlmA^I and RlmA^{II} would suggest that both subclasses of enzyme recognized their rRNA targets by very similar means. However, there must be differences in the manner by which the catalytic site in RlmA^I and RlmA^{II} lines up with the loop of hairpin 35 to distinguish how the two subclasses target bases that are displaced by three nucleotides in the rRNA. A first step towards understanding how the two subclasses of methyltransferases differentiate between their targets is to obtain a clear picture of the hairpin 35 structure in the conformation that is recognized by the methyltransferases.

Here we have investigated how RlmA^{II} recognizes its target in 23S rRNA. NMR spectroscopy was used to determine the solution structure of a 24 nt RNA oligonucleotide corresponding to hairpin 35 from 23S rRNA. Hairpin 35 was additionally investigated by chemical probing to determine whether its conformation in the isolated 24 nt oligomer is the same as within the larger transcript of domain II of the rRNA, and also within the intact 23S rRNA. After it was established that hairpin 35 folds independently of other 23S rRNA structures, the interaction of the *Streptococcus pneumoniae* RlmA^{II} methyltransferase with the 24 nt transcript of hairpin 35 was investigated.

Fig. 1. (A) Schematic representation of the secondary structure of the domains of *E. coli* 23S rRNA (Gutell *et al.*, 1994). Hairpin 35 loop in domain II and the peptidyl transferase centre in domain V of the rRNA are boxed. (B) Secondary structure of helix 33 (nucleotides 730–722 and 795–804), hairpin 34 (nucleotides 732–764) and hairpin 35 (nucleotides 768–792) in *S. pneumoniae* 23S RNA. The position of RImA^{II} methylation is at G780 (equivalent to G748 in the *E. coli* rRNA). (C) Sequence of the hairpin 35 used in the NMR study. Nucleotides present in the *E. coli* 23S rRNA structure are in bold.

The structure of a 24 nt RNA containing the conserved nucleotides of the loop of 23S rRNA hairpin 35 (Figure 1) was assayed by chemical modification under a range of pH and ionic conditions. The chemical reactivities of bases in the small oligonucleotide were compared with the reactivities of the same bases within *in vitro* transcribed domain II rRNA, within 23S rRNA and within functional 50S subunits. The stem section of the 24 nt RNA is stable at 37°C, while the N1 positions of the four adenines in the loop are accessible to dimethylsulfate (DMS), as are the two loop guanines to kethoxal (Figure 2). Neither magnesium ion concentrations (Figure 2A) nor changing the pH within the range 6.4–8.0 has measurable effects on the loop conformation or on the stability of the stem section at 37°C. Chemical probing of the hairpin 35 loop in domain II transcripts and in intact 23S rRNA showed the same modification pattern as in the isolated hairpin 35 transcripts (Figure 2B), indicating that the folding of the loop was independent of other regions in the rRNA.

For NMR purposes, the non-conserved *E.coli* base pair U744–A753 at the end of the stem adjacent to the loop was substituted for a C744–G753 pair found in *S.pneumoniae* 23S rRNA and many other Gram-positive sequences (Gutell *et al.*, 1994). Neither of these terminal base pairs changed the accessibility of the four adenine bases in the loop of hairpin 35 (Figure 2A), nor were the accessibilities of the N1/N7 positions of the loop guanines affected (not shown). The loop of hairpin 35 is highly conserved and the loop sequence shown in Figure 1 is identical to that found in both *E.coli* and *S.pneumoniae*.

The hairpin 35 oligonucleotide as a substrate for BImA^{II}

Methylation of guanine 748 by RlmA^{II} was investigated in a series of RNA transcripts containing hairpin 35. The transcripts ranged in size from the 24 nt fragment up to 726 nt domain II transcript and the intact 23S rRNA. A transcript of 92 nt containing the *E.coli* rRNA stem-loops 33, 34 and 35 (Hansen *et al.*, 2001) was methylated as efficiently as the intact 23S rRNA and the domain II transcript. Truncation of helices 34 and 35 reduced the rate of substrate methylation, and complete removal of helix 33 or helix 34 and the three-way junction between them greatly reduced methylation. The rate of methylation of the 24 nt transcript of hairpin 35 by RlmA^{II} is <3% that of the intact 23S rRNA.

These varied rates of methylation for the different RNA substrates correlate well with the results of the gel shift assays. An apparent dissociation constant of $5 \pm 3 \mu\text{M}$ was measured by a native gel mobility shift assay for a 74 nt RNA that includes part of helix 33, helices 34 and 35, and the three-way junction between the helices. A large molar excess of a non-substrate RNA (a stem with a UUCG tetra loop) was incapable of dissociating the complex between the 74 nt RNA and RImA^{II}, demonstrating the specificity of the interaction. A weaker interaction was formed with the 24 nt hairpin 35 RNA that could only be detected at high enzyme concentrations. This result indicates that the apparent dissociation constant for the 24 nt RNA is appreciably higher than $5 \mu\text{M}$, but we were

Fig. 2. (A) Chemical probing of the hairpin 35 oligonucleotide analyzed by primer extension. The hairpin loop structure is closed by either a U744–A753 base pair (H35-AU) or a C744–G753 base pair (H35-GC). Samples were probed in 20 mM PIPES pH 6.4, 200 mM NH₄Cl for 15 min with DMS. Samples 1 and 2 are unmodified controls; samples 1 and 3 contained no magnesium; sample 4 contained 1 mM MgCl₂; samples 2 and 5 contained 20 mM MgCl₂. The dideoxy sequence is shown on the right (CUAG). **(B)** Probing of the intact 23S rRNA, domain II transcript (Dom II RNA) and the hairpin 35 transcript with the C744–G753 base pair (H35-GC). Samples were modified with DMS or kethoxal in 20 mM HEPES pH 7.8; 200 mM NH₄Cl; 20 mM MgCl₂. Control lanes (unmod) show unmodified samples. **(C)** Probing of 23S rRNA and 50S subunits from *E. coli* IB10 (Björk and Isaksson, 1970) with DMS and DEP in the same buffer as (B). Samples were modified (+) with the reagents as indicated; no + represents unmodified controls. RNA were analyzed by primer extension using primer mfl1 (Liu *et al.*, 2000).

Table I. Structural statistics and atomic r.m.s. deviations

Total number of experimental restraints:	
Distance restraints	289
Dihedral restraints	77
Final distance and dihedral restraint:	
Violation energies (kcal/mol)	1.597 ± 0.642
R.m.s. deviation from experimental restraints:	
Distance restraints (Å)	0.0076 ± 0.0018
Dihedral restraints (°)	0.0185 ± 0.005
Deviations from idealized geometry:	
Bonds (Å)	0.03355 ± 0.00003
Angle (°)	0.0793 ± 0.0005
Impropers (°)	0.0378 ± 0.0033
Heavy atoms r.m.s. deviation (Å)	1.45 ± 0.48
All RNA	<SA> versus SA ^a

^a<SA> refers to the final 17 simulated annealing structures, SA to the average structure obtained by taking the average coordinates of the 17 simulated annealing structures best-fitted to one another. The 17 final structures did not contain distance violations of >0.1 Å or dihedral violations of >5°.

unable to accurately measure this value by the gel shift method.

Assignment of proton resonances

The solution structure of the hairpin 35 loop was characterized using the 24 nt RNA prepared by *in vitro* transcription from an oligonucleotide template (Figure 1C). Milligram quantities of unlabeled, ¹⁵N and ¹³C/¹⁵N-labeled RNAs were prepared, and complete ¹H assignments were obtained using standard heteronuclear (¹H, ¹⁵N, ¹³C, ³¹P) multidimensional NMR experiments and through-bond assignment strategies. All base-paired imino proton resonances in the stem regions were assigned via sequential nuclear Overhauser effects (NOEs) observed in 2D NOESY spectra and with ¹H/¹⁵N HSQC spectra. Watson–Crick N–H–N hydrogen bonds were directly observed in heteronuclear J(N,N)-HNN COSY experiments performed in H₂O (Dingley and Grzesiek 1998) and D₂O (Hennig and Williamson, 2000). In the hairpin loop, the G745 imino resonance was partially protected from solvent exchange and could be sequentially assigned in a 2D NOESY spectrum performed at 2°C (75 ms mixing time) and confirmed as a G imino in the ¹H/¹⁵N HSQC spectra at 2°C. The G748, U746 and U747 imino resonances were also partially protected from exchange with H₂O but could not be detected in the ¹H/¹⁵N HSQC spectra. The chemical shifts and NOE patterns observed for the RNA were not significantly affected by addition of 3 mM Mg²⁺ (data not shown), indicating that the structure is maintained under more physiologically relevant conditions.

Complete assignments for all the non-exchangeable proton resonances and their attached carbons were obtained using standard homonuclear 2D and heteronuclear 3D experiments.

Structure of the hairpin 35 loop

A total of 289 NOE distance restraints and 77 dihedral torsion restraints were obtained from the NMR data for the RNA loop. Structures were calculated using restrained molecular dynamics in a simulated annealing protocol.

The overall structure is well-defined by the NMR data (Table I), with a heavy atom root-mean-square (r.m.s.) deviation of 1.45 Å (Table I, Figure 3A) for the 17 structures.

The loop of hairpin 35 folds as a compact and stable structure (Figure 3B). The imino proton walk and the aromatic-H1' connectivities along the helical stem show that it extends into a sheared G745–A752 base pair, which is formed by the N2 amino group and N3 of G745 contacting the N7 and N6 amino group of A752, respectively (Figure 3C). In the loop, U746 and U747 are continuously stacked above G745 with a sharp turn occurring in the phosphodiester backbone between U747 and G748. The sharp foldback of the phosphodiester backbone is stabilized by a U-turn, where the N3 of U747 forms a potential hydrogen bond with the phosphate of A750, and the 2'OH of U747 forms a hydrogen bond with the N7 of A749. An intra-residue hydrogen bond is formed between the 2'OH group and the N3 nitrogen of G748. The four nucleotides, G748 to A751 (including the universally conserved nucleotides G748 and A750), are continuously stacked at the 3' side of the loop with their Watson–Crick faces and ribose-phosphate groups available for interaction with proteins.

Binding of RlmA^{II} methyltransferase induces chemical shift changes in the RNA

Observation of chemical shift changes upon binding of RlmA^{II} to hairpin 35 can be used to map the surface of the RNA contacted by the protein. The exchangeable proton resonances of the RNA in the presence of increasing concentrations of RlmA^{II} were monitored in 1D imino proton spectra. ¹H-¹⁵N HSQC spectra were also recorded for different concentrations of RlmA^{II}. At a protein:RNA ratio of 1:4, the resonance of G753 is slightly modified (data not shown) and upon further addition of RlmA^{II} protein the G753 resonance disappeared. No other chemical shift differences were observed in the stem. Addition of *S*-adenosylhomocysteine, an analog of the methyl donor *S*-adenosylmethionine, in 4-fold molar excess relative to the methyltransferase resulted in a broadening of the U754 peak. When the ratio of RlmA^{II} protein:RNA exceeded 1:4, the imino proton resonances of the entire RNA loop broadened appreciably at low temperature (5°C).

¹H-¹³C HSQC spectra of aromatic and ribose resonances were recorded in D₂O to determine whether the chemical shifts in the non-exchangeable RNA protons are changed by RlmA^{II} (Figure 4). Specific chemical shift changes were detected by binding of hairpin 35 to RlmA^{II} whereas no modification could be detected with a control RNA containing a stem–loop closed by a UUCG tetra loop (data not shown). At a 1:4 ratio of protein:RNA, the C2 and C8 resonances of A742, and the C8 resonances of G748, G745, A752, A751 and G753 were no longer visible (Figure 4 and Table II). Similarly, the C6 resonances of residues C744, U746 and the C5 resonances of U746, U747 were no longer detected on titration with RlmA^{II}. The RlmA^{II} methyltransferase did not affect the chemical shifts of other aromatic carbons. In the ¹H-¹³C HSQC spectrum displaying the C4' region of the RNA, all C4' resonances in the loop region (G745–A752) were affected by binding of RlmA^{II} (Table II). No effects were observed

Fig. 3. (A) Best-fit superposition of 17 final simulated annealing structures of the hairpin 35 loop RNA. The heavy atoms of the RNA have been superimposed. Bases are shown in blue and the backbone in light blue. (B) Stereo view of a single representative structure of the hairpin 35 loop RNA. All heavy atoms are displayed. Bases are colored in light blue with nitrogen and oxygen atoms in dark blue and red, respectively. Ribose-phosphate backbones are colored in yellow and phosphate oxygen in red. (C) Possible hydrogen bonds in the sheared G745–A752 base pair are represented by dashed lines.

in the other C4' resonances in the stem. Similar observations were made for some of the C2', C3' and C5' positions in the riboses within the loop when bound to RlmA^{II} (Table II). In the presence of 3 mM Mg²⁺, the methyltransferase enzyme caused the same pattern of broadening of proton resonances as that listed in Table II.

Discussion

NMR structure of hairpin 35 correlates with the chemical probing data

NMR can be applied to determine the precise structures of small RNA fragments that simulate functional sites from larger RNA molecules. The 24 nt RNA was designed to

Fig. 4. (A) ^1H - ^{13}C HSQC spectrum showing the aromatic H8-C8, H6-C6 and (B) $\text{H}2/\text{C}2$ correlation of the hairpin 35 loop RNA at 20°C in the free form RNA (blue lines) and with a ratio 0.25:1 protein:RNA (purple lines). Nucleotides for which NMR signals broaden upon binding to RlmA^{II} are highlighted with a star.

represent the structure of hairpin 35 in 23S rRNA. Hairpin 35 is an important component of the binding site for macrolide drugs, and the hairpin loop is modified by RlmA^{II} methyltransferases, contributing to macrolide resistance. First of all it was established whether the structure of the loop of the 24 nt RNA truly represents the conformation in 23S rRNA that is recognized by the RlmA^{II} methyltransferases. This is indeed the case as the reactivity pattern of the 24 nt model RNA towards chemical probing reagents was indistinguishable from that observed for the loop of hairpin 35 within 23S rRNA. Furthermore, substitution (for NMR purposes) of the non-conserved U744–A753 base pair in the stem for the C744–G753 base pair found in *S. pneumoniae* 23S rRNA did not affect the overall conformation of the loop.

The NMR structure of hairpin 35 loop correlates well with the chemical probing data. The four adenine residues A749–A752 are continuously stacked in the NMR structure with each of the N1 positions accessible to the solvent. Nucleotide A752 forms a sheared base pair with G745, although this does not involve the N1 position of the adenine. Consistent with this, the N1 position of each of these adenines is accessible to DMS modification in the free RNA. The loop of hairpin 35 is highly conserved, and the loop structure determined here may be found in hairpin 35 of many 23S RNA molecules. In some 23S rRNA sequences, G is found instead of U747 at the U turn motif of hairpin 35. In GNRA tetraloops, a sharp turn equivalent to the U-turn is stabilized by a G amino-phosphate hydrogen bond (Heus and Pardi, 1991).

Table II. List of the specific chemical shift changes that were detected after binding of RlmA^{II} to hairpin 35

Resonances	Nucleotides
C8/C6	A742, C744, G745, U746, G748, A751, A752, G753
C5/C2	U746, U747, A752
C1'	G745–A752
C2'	A749
C3'	U747, A749
C4'	G745–A752
C5'	G745, U746, U747

The same effects were observed with and without magnesium ions.

Fig. 5. (A) View of hairpin 35 solution structure. (B) View of the crystal structure of tRNA^{Phe} anticodon stem-loop. All heavy atoms are displayed. Bases are colored in light blue with nitrogen and oxygen atoms in dark blue and red, respectively. Hydrogen bonds that contribute to stabilize the U-turn conformation are represented as dashed lines.

The loop of hairpin 35 is structurally similar to a tRNA anticodon loop

The loops of both hairpin 35 and tRNA have a stretch of stacked nucleotides, corresponding to G748–A752 in hairpin 35 and positions 34 to 38 in tRNA (Figure 5). Both structures have a U-turn motif, where a sharp change in the direction of the RNA backbone occurs between nucleotides 33/34 of tRNA and nucleotides 747/748 of hairpin 35. Comparison of the chemical reactivity to DMS and diethylpyrocarbonate (DEP) of the tRNA anticodon stem-loop (Shelton *et al.*, 2001) and hairpin 35 (Figure 2C) revealed some similarities and further support the result that both RNA molecules have a similar fold. Anticodon loops contain seven nucleotides, none of which is involved in intramolecular base pairing, while the hairpin 35 is composed of eight nucleotides with six unpaired bases and a non-canonical G745–A752 base pair. Similar loop structures containing a U-turn motif have been described, including a six nucleotide adenosine-rich loop in HIV (Puglisi and Puglisi, 1998), and in stem-loop IIa of yeast U2 RNA (Stallings and Moore, 1997). In tRNA, position 37 contains a hyper-modified purine, which does not allow base pairing and conserves a conformation with seven unpaired bases (Sussman *et al.*, 1978). Similarly, in hairpin 35 the corresponding nucleotide at A751 does not form a base pair with U746, although here there are no modifications that would prevent such an interaction. Substitution of U746 for a pseudouridine, a modification found in *E. coli* 23S rRNA (Wrzesinski *et al.*, 1995), did not affect the NMR structure (data not shown).

Fig. 6. Two different views of the hairpin 35 loop RNA indicating nucleotides involved in the interaction with RlmA^{II} methyltransferase. Nucleotides for which NMR signals broaden upon binding to RlmA^{II} are highlighted in yellow.

U746 of hairpin 35 and U32 of tRNA^{Phe} are modified to form pseudouridine by the same pseudouridyl synthetase (Wrzesinski *et al.*, 1995). It was proposed that the sequence identity between nucleotides U746 to A752 and the equivalent segment U32 to A38 in the tRNA^{Phe} anticodon is responsible for the dual specificity of the enzyme. The data presented here indicate that the higher-order structures are also strikingly similar with the modified U at the 5'-side of the U-turn nucleotide, and this is probably just as pertinent in explaining why the hairpin 35 and the tRNA anticodon loops are modified by the same pseudouridyl synthetase.

Surface of the hairpin 35 loop contacting RlmA^{II}

Clear changes occur in the NMR spectra of the hairpin 35 oligonucleotide upon addition of the RlmA^{II} methyltransferase (Figure 6). These changes appear specific for the RlmA^{II}–hairpin 35 interaction as no chemical shift changes are seen when a control RNA containing a UUCG tetra loop is substituted for the hairpin 35 RNA. However, RlmA^{II} is only marginally effective at methylating substrates as small as the 24 nt hairpin loop oligonucleotide under the methylation conditions used here. We put this down to the different conditions used for the NMR measurements and the methylation reaction, where the former conditions require substrate and enzyme concentrations far higher than the latter. The slow rate of methylation for the 24 nt hairpin loop oligonucleotide probably results from its low affinity for the enzyme, which was confirmed by the gel shift assay. The larger RNA substrates, such as the 74 nt RNA, displayed dissociation constants of ~5 μ M, which favor a more stable enzyme interaction, and are consistent with the efficient methylation of these substrates under the conditions we used here. The NMR data also agrees with a low affinity of hairpin 35 for the enzyme, as the observed broadening of specific proton resonances upon addition of RlmA^{II} is characteristic of an intermediate exchange regime. Methylation studies of small RNA substrates by RlmA^I, a related methyltransferase, showed that whilst the

essential determinants for the reaction lie in hairpin 35, structures within helices 33 and 34 are additionally required for efficient methylation (Hansen *et al.*, 2001). This is also the case for the RlmA^{II} methyltransferases, and therefore our NMR data, while revealing real effects of the RlmA^{II} methyltransferase interaction with the hairpin 35 loop, only represent a subset of the RNA contacts that are required for effective binding of the enzyme.

Most of the imino protons of the loop nucleotides, which are in fast exchange with the solvent, do not represent a good structural probe for the NMR studies. Indeed, only the imino proton chemical shift of G753 was affected by binding to RlmA^{II}. The complex was therefore studied by observing the non-exchangeable proton in the RNA loop. Upon gradual addition of protein, intermediate exchange dynamics between free and RlmA^{II}-bound RNA caused selective line broadening of the non-exchangeable proton resonances, in agreement with the low affinity of the RNA for the enzyme. The stem of the RNA oligonucleotide remains mostly unaffected by binding to the protein, in contrast to the ribose resonances and some aromatic resonances of the loop that gradually disappear from the ¹H-¹³C HSQC spectrum (Figure 6). The results suggest that a large surface of the loop is involved in or affected by the binding of the RNA to the methyltransferase.

Major structural rearrangement of the hairpin 35 loop on assembly of the 50S subunit

The consistency of the chemical modification and NMR data strongly suggest that the NMR structure of the hairpin 35 oligonucleotide represents the conformation of this region within the free 23S rRNA. However, after 23S rRNA has assembled into functional 50S subunits, the hairpin 35 loop displays a conformation that is strikingly different (Figures 2C and 7). This indicates that a major structural rearrangement occurs in the RNA, which can be seen to be linked to interaction with ribosomal protein L22 and portions of domain V of 23S rRNA (Gregory and Dahlberg, 1999; Ban *et al.*, 2000). The binding of the β -hairpin of L22 to the hairpin 35 loop results in the

Fig. 7. View of the hairpin 35 structure in solution (A) and of hairpin 35 loop region of 23S rRNA from the crystal structure of *Haloarcula marismortui* (Ban *et al.*, 2000) (B). All heavy atoms are displayed. Bases are colored in light blue with nitrogen and oxygen atoms in dark blue and red, respectively. Ribose-phosphate backbones are colored in yellow and phosphate oxygen in red. The tip of the β -hairpin of r-protein L22 interacting with the hairpin 35 loop is represented in purple. The conformations of hairpin 35 and r-protein L22 are essentially the same in the *Deinococcus radiodurans* 50S subunit (Schlünzen *et al.*, 2001), which is presently the only other high resolution 50S structure available.

opening of the tip of the loop with stabilization of bulged nucleotides by extensive base stacking with nucleotides from domain V. Consistent with this, in the assembled 50S subunit, the N1 positions of adenines A749 and A750 become inaccessible to DMS and the N7 positions of A749–A753 become unreactive to DEP, whereas these positions are readily modified in the free RNA (Figure 2C). The N1 positions of A751 and A752 remain reactive to DMS in the assembled ribosome, and there is evidence that macrolide antibiotics interact here (Moazed and Noller, 1987; Hansen *et al.*, 1999; Xiong *et al.*, 1999; Poulsen *et al.*, 2000). The differences in reactivity patterns support the notion that the folding of this region of 50S subunit is a cooperative process that requires RNA–RNA and protein–RNA interactions leading to distortion of the hairpin 35 loop observed within the ribosome.

Materials and methods

Chemical probing of the RNA structure

The structures of various RNAs, each containing hairpin 35 from 23S rRNA, were probed under a range of pH and ionic conditions. Ten picomoles of the hairpin 35 RNA (plus 30 pmol carrier tRNA), 4 pmol of a transcript of 23S rRNA domain II, or 1 pmol of 23S rRNA were renatured in 100 μ l of 20 mM HEPES pH 7.0–8.0 (or 20 mM PIPES pH 6.4–6.9); 50–200 mM NH_4Cl ; 0–20 mM MgCl_2 by warming at 50°C for 5 min followed by incubation at 30°C for 10 min. The hairpin 35 transcripts used for chemical probing had either a U–A or a C–G as the closing 744–753 base pair at the end of the stem, and a 16 nt extension corresponding to the *E. coli* 23S rRNA sequence at 761–776 to facilitate primer extension analysis of the RNAs. Samples were modified with either DMS, 2 μ l of a 1:6 (v/v) dilution in 96% ethanol, 5 μ l DEP or kethoxal (3 μ l of a 40 mg solution in 600 μ l 33% ethanol). After incubation at 30°C for 10–30 min, reactions were stopped and rRNA was recovered as described by Moazed *et al.* (1986). Sites of modification were analyzed by primer extension (Stern *et al.*, 1988).

Methylation reactions in vitro

23S rRNA (1 pmol) and T7 RNA transcripts (4 pmol) containing the hairpin 35 sequence (Hansen *et al.*, 2001) were renatured in 100 μ l buffer containing 20 mM HEPES pH 7.5, 100 mM NH_4Cl , 10 mM MgCl_2 , 10% glycerol and 6 mM β -mercaptoethanol by heating at 50°C for 5 min followed by 10 min at 37°C. *S*-adenosylmethionine was added to a final concentration of 1 mM together with 0.1 pmol *S. pneumoniae* RlmA^{II} (see

below) to start the methylation reaction. Methylation reactions were stopped and RNA recovered as described previously (Hansen *et al.*, 2001).

Methylation at G748 was quantified by a procedure adapted from Sigmund *et al.* (1988), using a 5'-³²P-labeled primer complementary to the A750–G763 region of the RNA and was extended with reverse transcriptase (Life Sciences) using a mixture of 100 μ M each of ddATP, dCTP and dTTP. Extension terminates directly before G748 on methylated rRNA, or at U747 on unmethylated rRNA due to incorporation of the dideoxynucleotide. Extension products were analyzed on 13% polyacrylamide/7 M urea gels, and the gel band intensities were measured by PhosphorImager scanning (Storm 840, Molecular Dynamics). The degree of methylation was calculated from the intensities of the U747 and G748 bands (Hansen *et al.*, 2001).

Gel shift assay

Apparent dissociation constants for RNA–RlmA^{II} complexes were estimated by a native gel mobility shift assay (Batey and Williamson, 1996). RNA constructs included a 74 nt RNA consisting of hairpin 35, hairpin 34 with a UUCG tetra loop replacing the original hairpin loop, and a version of helix 33 truncated to 6 bp (Figure 1B); the 24 nt (Figure 1C); several intermediate-sized RNAs (Hansen *et al.*, 2001) and a control RNA consisting of a stable stem and tetra loop. RNAs were at 0.1 μ M per sample, and were radioactively labeled with ³²P; the concentration of RlmA^{II} in the binding assays was varied from 0.1–100 μ M. Reaction mixtures were incubated at room temperature for 15 min in 10 μ l of 10 mM sodium phosphate (pH 6.4), 0.5 mM dithiothreitol (DTT) and 0.01% Nonidet P-40, before complexes were separated from the free RNA on 8% polyacrylamide non-denaturing gels. Binding specificity was controlled by addition of a 100-fold molar excess of the unlabeled control RNA. Data were analyzed using the MC-Fit program (Dardel, 1994) to deduce apparent dissociation constants.

Preparation of the RNAs for NMR

Milligram quantities of the RNA (24 nucleotides) were prepared unlabeled, and uniformly ¹⁵N- and ¹³C/¹⁵N-labeled by *in vitro* transcription from an oligonucleotide template containing a 2' OmeG in position 2 (Kao *et al.*, 1999), and were purified as described by Puglisi and Wyatt (1995). After electroelution and ethanol precipitation, the resuspended RNA was then dialyzed for 48 h against the buffer used for the NMR experiment in a microdialysis apparatus with a 3500 mol. wt cut-off membrane. Labeled NTPs were isolated from *E. coli* bacteria strain MRE600 grown in rich ¹³C/¹⁵N media (Martek CN9) or M9 media containing ¹⁵N ammonium chloride. Labeled NMPs were purified and converted to NTPs as Batey *et al.* (1995) and Nikonowicz *et al.* (1992). The hairpin 35 RNA containing a pseudouridine at position 746 was purchased from Dharmacon Research, Inc. (Lafayette, CO).

Expression and purification of RlmA^{II}

The *S. pneumoniae* *rlmA*^{II} gene was inserted into the pGEX2T gene fusion vector (Smith and Johnson, 1988). *Escherichia coli* BL21 was transformed with the recombinant plasmid, and transformants were grown at 37°C to an A₆₀₀ of 0.4 in LB medium containing ampicillin at 100 mg/l. Addition of IPTG to 1 mM induced expression of the RlmA^{II} methyltransferase as a fusion protein with glutathione *S*-transferase. After a further 4 h incubation, cells were pelleted and resuspended in a buffer at pH 7.3 (150 mM NaCl, 16 mM Na_2HPO_4 , 4 mM NaH_2PO_4) containing 0.2 mM phenylmethyl sulfonyl fluoride. Cells were lysed on ice by sonication and centrifuged at 10 000 r.p.m. for 10 min at 4°C. The supernatant was applied to a glutathione agarose column (Sigma) equilibrated with the phosphate buffer. After washing with 50 mM Tris–HCl buffer pH 8.0, the fusion protein was eluted with the same Tris–HCl buffer containing 5 mM reduced glutathione (Sigma). Twenty-four milligrams of the purified fusion protein was incubated overnight at 25°C with 33 μ g thrombin (Sigma) in elution buffer containing 150 mM NaCl. After dialysis, the mixture was loaded onto a DEAE–Sephacrose column (Pharmacia) equilibrated in 20 mM Tris–HCl buffer pH 7.0 and 5 mM DTT, and the protein was eluted with a salt gradient. The protein was redialyzed and was then applied to a carboxymethyl–Sephacrose column (Pharmacia) equilibrated in 10 mM sodium phosphate pH 6.4 and 5 mM DTT. The RlmA^{II} was eluted with a salt gradient and was finally dialyzed against 10 mM sodium phosphate pH 6.4, 5 mM DTT.

For the NMR studies, the protein was concentrated in 10 mM sodium phosphate buffer at pH 6.4 and 5 mM DTT, using centricons (Millipore). For ¹H–¹³C HSQC, H₂O was exchanged to D₂O using repeated concentration and dilution with D₂O buffer.

Proton and heteronuclear NMR

NMR experiments were recorded at 800 MHz on a Bruker DRX spectrometer equipped with triple resonance, z -gradient probes. NMR experiments involving ^{31}P were recorded on a Bruker DRX spectrometer at 600 MHz. NMR data were processed using XWINNMR, Aurelia (Bruker) and Gifa (Pons *et al.*, 1996) software packages. NMR experiments were performed in Shigemitsu NMR tubes containing 280 μl 10 mM sodium phosphate (pH 6.4), with 3 mM MgCl_2 for some experiments. The concentrations of unlabeled RNA, uniformly ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labeled RNA ranged from 1 to 4 mM. ^{15}N and ^{13}C chemical shifts were referenced with the known chemical shifts of the ribose carbon or base nitrogen from the 5' and 3' end regions (Yoshizawa *et al.*, 1998).

^1H , ^{13}C , ^{15}N and ^{31}P assignments were obtained using standard homonuclear and heteronuclear methods. NMR data were acquired at either 20 or 30°C and data for exchangeable protons were collected either at 2, 5 or 15°C. Solvent suppression for samples in 90% $\text{H}_2\text{O}/10\%$ D_2O was achieved using the WATERGATE sequence (Piotto *et al.*, 1992; Sklenar *et al.*, 1993). The residual HDO resonance in D_2O was suppressed using low power presaturation. 2D-NOESY spectra in 95% $\text{H}_2\text{O}/5\%$ D_2O were acquired with mixing times of 50, 75, 150 and 300 ms. The hydrogen bonding patterns of the base pairs were determined from analysis of NOESY spectra in H_2O at different mixing times and directly observed from heteronuclear J(N,N)-HNN COSY experiments performed in 90% $\text{H}_2\text{O}/10\%$ D_2O (Dingley and Grzesiek, 1998) and 100% D_2O (Hennig and Williamson, 2000). NOESY spectra with mixing times of 50, 150, 250 and 400 ms in D_2O were measured at 15, 25, 30 and 35°C.

Heteronuclear NMR spectra were measured at 30°C in 99.9% D_2O , with the exception of ^1H - ^{15}N HSQC experiments that were acquired at 2, 5 and 15°C in H_2O . ^{13}C and/or ^{15}N decoupling during the acquisition time was achieved using the GARP composite pulse sequence. The assignment of the non-exchangeable protons of the labeled RNA was completed using constant-time HSQC (Santoro and King, 1992), 3D-HCCH-TOCSY and 3D-NOESY-HMQC (100 and 200 ms mixing times) (Clare *et al.*, 1990). Sequential connectivities between nucleotides in the hairpin 35 loop RNA were obtained by HP-COSY experiments (Sklenar *et al.*, 1986) at 30°C, HCP experiments (Marino *et al.*, 1995) and HCN experiments (Riek *et al.*, 2001). The H2 protons of adenines were unambiguously assigned for the labeled RNAs by correlation of the H2/H8 resonances in 2D-HCCH-TOCSY experiments (Legault *et al.*, 1994; Marino *et al.*, 1994).

Distance and dihedral restraints for structure calculation

Distance restraints involving non-exchangeable RNA protons were derived from visual inspection of cross-peak intensities in 50, 150, 250, 400 ms NOESY experiments and 3D-NOESY-HMQC spectra. The H5/H6 cross-peak of pyrimidines was used as an internal standard. NOEs were classified into four distance bound ranges: strong 0–3 Å, medium 0–4 Å, weak 0–5 Å and very weak 0–6 Å. In several instances, very weak NOE restraints observed in the loop region were loosened to 0–7 Å. The appropriate pseudoatom distance corrections were used.

RNA dihedral restraints were assigned following a previously described strategy (Allain and Varani, 1995). β dihedral angles were restrained from estimates of the $^3J_{\text{P-H5'}}$, $^3J_{\text{P-H5''}}$ and $^3J_{\text{P-C4'}}$ coupling constants from the HP-COSY experiment on the unlabeled RNA. ϵ was also restrained from estimates of $^3J_{\text{H3'-P}}$, $^3J_{\text{C2'-P}}$ and $^3J_{\text{C4'-P}}$ from the HP-COSY and HCP experiments. The ϵ dihedral angles were constrained to $210 \pm 30^\circ$ (*trans*) or $260 \pm 20^\circ$ (*gauche*⁻) or $235 \pm 55^\circ$ (when the *trans* or *gauche*⁻ conformations could not be distinguished).

The γ dihedral angles were constrained where possible using estimates of $^3J_{\text{H4'-H5'}}$ and $^3J_{\text{H4'-H5''}}$ coupling constants from the ^{31}P decoupled DQF-COSY. For a *gauche*⁺ conformation, γ was constrained to $55 \pm 20^\circ$. The ribose sugar pucker was estimated from analysis of the H1'-H2' coupling constants in the ^{31}P decoupled DQF-COSY spectrum. Nucleotides with a H1'-H2' coupling constant of >8 Hz in the COSY spectrum were classified as C2'-*endo* ($\delta = 150 \pm 30^\circ$). Nucleotides with no COSY and TOCSY cross-peaks between the H1'-H2' protons ($J < 3\text{Hz}$) were classified as C3'-*endo* ($\delta = 85 \pm 20^\circ$). Some nucleotides had weak H1'-H2' cross-peaks in the TOCSY spectrum, but not in the COSY. When a mixed population of C2'/C3'-*endo* conformations was observed, the ribose pucker for these nucleotides were restrained in the range of the C2'+ C3'-*endo* conformations during molecular dynamics.

Structure determination

Structures were calculated using a simulated annealing protocol (AMBER force field) within the Insight II package (Biosym Technologies, San Diego, CA) as previously described (Puglisi and Puglisi, 1998). Fifteen percent of the structures converged, as based on

restraint violation energies, and 30 of them were collected to be further refined with the final set of restraints. The lowest energy non-converged structures had total energies >4 standard deviations for the converged structures. The 17 final structures shown in Figure 4A had the lowest total energy and restraint violation energies. For the hairpin 35 loop RNA, a total of 289 distance restraints were used including 81 intranucleotide RNA restraints, 164 internucleotide RNA, 44 base pair hydrogen bonding restraints; no hydrogen bonding restraints were used for non-canonical base pairs. A total of 77 experimental dihedral restraints were used. The final force constants for distance restraints were 40 kcal/mol. Base pairing hydrogen bond and dihedral restraints final force constants were set to 250 kcal/mol and 60 kcal/mol, respectively. All color figures were generated with the program InsightII (Biosym Technologies, San Diego, CA).

Coordinates

The coordinates for the ensemble of 17 structures for the hairpin 35 RNA have been deposited to the Protein Data Bank with accession number 1MT4.

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