coupling constraints should make it possible to structurally relate distant regions of the molecule. In this way the addition of dipolar coupling data should lead to improvements in both the local and global conformations of proteins and nucleic acids determined by solution NMR techniques.

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[16] Biochemical and Nuclear Magnetic Resonance Studies of Aminoglycoside–RNA Complexes

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Introduction

Nuclear Magnetic Resonance (NMR) spectroscopy has become a powerful tool for studying RNA–ligand interactions. Our laboratory has studied complexes formed between aminoglycoside antibiotics and an RNA oligonucleotide that mimics the ribosomal RNA target of these drugs. Size limitations of approximately <30,000 molecular weight (100 nucleotides) prevent the study of the whole biological system, the ribosome, by NMR. Aminoglycoside antibiotics bind both with high affinity and specificity to short oligonucleotide sequences, which makes the system ideal for NMR. Structural studies have revealed features of the drug and RNA required for binding.

The aminoglycosides are effective antibiotics because they bind to prokaryotic ribosomes more tightly than to eukaryotic ribosomes. Aminoglycoside antibiotics bind to the aminoacyl-tRNA site (A site) of 16S rRNA on the 30S subunit and interfere with protein synthesis by inducing codon misreading and inhibiting translocation. Structural studies of an aminoglycoside–RNA complex provide insights into miscoding and reveal the origins of specific aminoglycoside binding to prokaryotic ribosomal RNA, thus leading to the possibility of using the three-dimensional (3-D) structure of


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the complex for structure-based drug design. This article focuses first on how to use biochemical methods to identify a minimal RNA sequence that mimics the aminoglycoside binding site on the ribosome, then on how to apply NMR spectroscopy to identify the site of interaction of the antibiotic on the oligonucleotide and subsequently determine the 3-D structure of the antibiotic–RNA complex.

Choice and Design of Antibiotic–RNA Oligonucleotide Complex

Several factors made the aminoglycoside–rRNA complex amenable to NMR study. First, the interaction of aminoglycosides with the ribosome had been previously characterized biochemically. Second, the site of interaction of the drug is localized to a small region of the 16S rRNA. Third, this region of rRNA does not appear to interact with ribosomal proteins. These factors suggested that the local structure of this region of rRNA could be maintained in an oligonucleotide in the absence of any of the ribosomal proteins.

Before NMR studies were started, it was necessary to determine if the model system was an accurate representation of the aminoglycoside–rRNA interaction. Figure 1A (see color insert) shows the secondary structure of 16S rRNA where the aminoglycoside antibiotic paromomycin binds and where mRNA decoding occurs. Figure 1B presents the oligonucleotide designed to mimic binding of the antibiotic. Because large quantities of sample are required for NMR, 2 G–C base pairs were added to the 5' end to enable high yield of RNA by in vitro transcription with T7 RNA polymerase. An ultrastable [5' UUCG 3'] tetraloop was also added to help ensure that the oligonucleotide forms the desired secondary structure with two 4-bp stems on either side of the asymmetric internal loop. Chemical probing experiments identical to the type used to characterize the drug–ribosome interaction were used to assay the interaction of the antibiotic with the oligonucleotide. Chemical probing and primer extension analysis of oligonucleotides requires that several changes be made to the standard ribosome modification experiments. These methods are outlined in the following section.

Methods

**Chemical Modification**

Chemical modification reactions are performed on the oligonucleotide in a buffer similar to that used for characterizing the interaction of the ribosome with aminoglycoside antibiotics. The duration of modification and/or concentration of modifying reagent must be adjusted for each oligonucleotide construct. It is important to ensure that, on average, there is no more than one modification on each oligonucleotide molecule. This can be estimated following primer extension as follows: if the band corresponding to full-length oligonucleotide is more intense than the sum of all reverse transcriptase stops below it, then the RNA has not been overmodified.

In the case of the A-site oligonucleotide, modification reactions (20 µl) are performed in 80 mM potassium cacodylate, pH 7.0, with 75 nM RNA oligonucleotide. To ensure proper folding, the RNA is heated at 90 ° for 1 min then placed on ice for 5 min. Antibiotics (Sigma, St. Louis, MO) are added and the modification performed by addition of 1 µl dimethyl sulfate (1:10 dilution in ethanol, final dilution 1:200) followed by incubation at room temperature for 5 min. Reactions are stopped by addition of 1/4 volume 1 M Tris–acetate, pH 7.5, 1.5 M sodium acetate, 1 M 2-mercaptoethanol followed by ethanol precipitation. To allow detection of modifications at the N-7 of guanine, sodium borohydride reduction and aniline-induced strand scission are performed as described except that lyophilization steps are replaced by phenol extraction and ethanol precipitation.

Modified RNA is resuspended in 10 µl 1 M Tris-HCl, pH 8.2. On addition of 10 µl of freshly prepared 0.2 M sodium borohydride, the samples are incubated on ice in the dark for 30 min. The reaction is quenched by addition of 300 µl 0.3 M sodium acetate followed by ethanol precipitation. Pellets are dissolved in 20 µl 1.0 M aniline/acetate, pH 4.5, followed by incubation in the dark for 20 min at 60 °. The reaction is terminated by addition of 110 µl 0.3 M sodium acetate, pH 5.5, and 110 µl phenol/chloroform/isoamyl alcohol (25:24:1) followed by vigorous mixing and centrifugation. The RNA is concentrated by ethanol precipitation of the aqueous phase and pellets washed with 100 µl 70% ethanol.

The RNA is resuspended in water (~1 pmol/µl) and primer extension is performed as described with the following modifications: (1) 5'-labeled primer is annealed at a 2-fold excess to RNA, (2) 4 µl of 1 mM dNTPs is added to each tube prior to addition of reverse transcriptase, and (3) five units (0.2 µl) of avian myeloblastosis virus (AMV) reverse transcriptase

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FIG. 1. (A) Secondary structure of *Escherichia coli* 16S rRNA. The A-site region is boxed. Nucleotides that are conserved in all 16S-like rRNAs are indicated in green. (B) Sequence of the A-site oligonucleotide used in NMR studies. Nucleotides critical for high-affinity binding of the aminoglycoside paromomycin to the oligonucleotide are indicated in red.
Fig. 4. Representative structures of (A) the free form and (B) the paromomycin-bound form of the A-site oligonucleotide. Nucleotides of the aminoglycoside binding site are shown in blue. All other nucleotides are green. Paromomycin is tan.

Fig. 5. Superposition of the paromomycin (black)- and gentamicin (red)- A-site RNA structures. Rings I and II of the antibiotics bind in a similar manner and induce the same conformational change in the RNA. In paromomycin, ring III is linked to the 5 position of ring II. Gentamicin has ring III linked to position 6 of ring II.
(Seikagaku, Ijamsville, MD) per tube was used in the extension. These changes decrease the background of spontaneous reverse transcriptase stops. A primer annealing site must be present at the 3' end of the oligonucleotide. In the case of the A-site oligonucleotide, a 17-nucleotide sequence was added to the original construct. Priming sites as short as 10 nucleotides may be used. Additional nucleotides are not required if one does not wish to monitor nucleotides near the 3' end of the molecule. Extension is performed for 30 min to 1 hr, and the reaction is stopped by addition of 20 \mu l 0.1 M sodium hydroxide followed by incubation for 15 min at 90° to digest remaining RNA. For RNA molecules up to 50 nucleotides, failure to treat with base prior to electrophoresis results in a smearing of bands. The DNA is precipitated and pellets washed with 70% (v/v) ethanol. DNA is suspended in 10 \mu l 7 M urea loading dyes, heated to 90° for 1–2 min, and 2.5 \mu l is loaded on a 20% (w/v) polyacrylamide (19:1 acrylamide:bis acrylamide) 7 M urea gel.

Comparison of Model System with Ribosome

Because the same chemical probing experiments were used to study the interaction of paromomycin with the A-site oligonucleotide and the ribosome, the results can be directly compared. Paromomycin causes protections of the same set of nucleotides at the same drug concentration on both the A-site oligonucleotide and the ribosome. Data from chemical probing experiments in which the antibiotic is titrated over the relevant concentration range can be used to determine an approximate \( K_D \) for the interaction of the drug with both the oligonucleotide and the ribosome. Band intensities are converted to fraction of RNA bound to antibiotic. These experiments indicated a \( K_D \) for the interaction of paromomycin with the A-site RNA of \( \approx 0.2 \mu M \), which is similar to that determined for the paromomycin–ribosome interaction by equilibrium dialysis.

Mutant Oligonucleotides

The specificity of the aminoglycoside antibiotic–A-site RNA interaction is studied by making mutations in the RNA sequence. For a short oligonucleotide, such as the A-site RNA, this is simply a matter of synthesizing appropriate DNA templates to be used for \textit{in vitro} transcription. The advantage of performing mutational analysis on the oligonucleotide is that it is a strictly \textit{in vitro} assay for the rapid screening of large numbers of mutants. Chemical footprinting experiments as a function of antibiotic concentration can be performed on the mutant oligonucleotides to determine an approxi-

mate binding constant. Nucleotides critical for the antibiotic–A-site RNA interaction were identified using this approach (Fig. 1B). Once interesting mutants in the oligonucleotide have been identified, these can be introduced in vivo.

It is possible to introduce mutations into plasmid-encoded copies of the 16S rRNA. We observed a direct correlation between nucleotides critical for the binding of paromomycin to the A-site oligonucleotide and to the ribosome, demonstrating the validity of the model system.

**Purification of RNA Sample for NMR Studies**

Large quantities of both antibiotic and RNA oligonucleotide are required for structural studies. Many antibiotics can be purchased at sufficient purity for low cost from chemical companies, but the RNA oligonucleotide must be synthesized and purified. The methods for synthesis and purification of the RNA NMR sample have been described elsewhere. After synthesis and purification of the RNA oligonucleotide, the NMR sample is dialyzed into buffer. A typical NMR buffer is 10 mM sodium phosphate at pH 6.0–7.0. Phosphate is commonly used because it lacks nonexchangeable protons that would interfere with the NMR spectrum of the RNA oligonucleotide and is a good buffer near neutral pH. Ideally, the concentration of the RNA in the NMR tube should be ~3 mM. The duration of an experiment can be greatly reduced with highly concentrated samples; to achieve an equivalent signal-to-noise ratio (SNR) with a sample half as concentrated as another, the NMR experiment needs to be run four times as long. However, some RNA oligonucleotides aggregate or dimerize at high concentrations, so spectra for these samples need to be acquired at lower concentrations or different sample conditions.

**13C/15N-Labeled RNA Samples**

A sample uniformly labeled with 13C and 15N is critical for high-resolution structure determination of RNA–antibiotic complexes. Complete assignment of the NMR spectrum of the RNA is only possible with heteronuclear experiments involving 13C or 15N or both. Labeling is accomplished at only moderate expense by initially isolating 13C- and 15N-labeled ribonucleotides from bacteria grown in media with 13CH3OH as the sole carbon source and 15NH4SO4 as the only source of nitrogen. The labeled nucleotides are purified using published protocols. Overall, approximately 50 mg of

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labeled NTPs can be isolated per liter of cell culture. Because the media contains 0.8 g of $^{13}$CH$_3$OH per liter, the NTP yield is approximately 62 mg NTPs/1 g $^{13}$CH$_3$OH.

Labeled nucleotides can be used in place of unlabeled nucleotides in the \textit{in vitro} transcription reaction. Yield of labeled RNA from the transcription reaction is often lower than the yield of unlabeled RNA, because the molar ratio of the four labeled NTPs is not equivalent. However, the transcription reaction can be optimized by titrating all components of the reaction to make the labeled NTPs the limiting reagent. An NMR sample can thus be purified from as little as 100 mg of NTPs for a small RNA molecule (<20 nucleotides) that synthesizes well. The cost of sample preparation may increase by as much as 10- to 15-fold for larger molecules and sequences that synthesize poorly. Heteronuclear NMR experiments that take advantage of the additional spin 1/2 nuclei are used to assign the proton NMR spectrum of the labeled RNA oligonucleotide. In addition, assignment of nuclear Overhauser effects (NOEs) that are overlapped in the $^1$H NOESY spectra is easier in a 3-D NOESY experiment with separation by carbon or nitrogen chemical shift in the third dimension.

\textit{NMR Assignments of RNA}

The first step after sample purification is to assign proton resonances of both the RNA and the antibiotic in their respective free forms. Characterization of the free RNA and antibiotic by NMR facilitates analysis of the RNA–drug complex, and possible conformational changes that occur on complex formation. Initial resonance assignment of the RNA in the absence of antibiotic is accomplished through application of well-established homonuclear and heteronuclear NMR experiments.\textsuperscript{12-15} Assignment strategies for nonexchangeable protons, which include the protons on the ribose sugar except for the 2'-OH, the H-5 and H-6 protons on pyrimidine bases, the H-8 of purine bases, and the H-2 proton on the adenosine base, have been discussed elsewhere.\textsuperscript{13-15}

\textit{Exchangeable Proton Assignment}

Recent NMR developments have greatly aided through-bond assignment of base exchangeable protons. Correlation experiments that apply

heteroTOCSY pulse schemes in water on uniformly \(^{13}\text{C}/^{15}\text{N}\)-labeled samples enable resonance assignment of all base protons that do not exchange rapidly with water. HeteroTOCSY experiments correlate exchangeable and nonexchangeable base protons: guanosine NH (imino) to H-8,\(^{16-18}\) uridine NH (imino) to H-6,\(^{17,18}\) cytidine NH\(_2\) (amino) to H-6,\(^{19}\) and adenosine NH\(_2\) (amino) to the H-2 and H-8.\(^{18,20}\) Each of these experiments is valuable for assigning the water exchangeable resonances on the bases.

An example of guanosine imino proton (H-1) to H-8 proton correlation is shown in Fig. 2. This experiment was performed on a uniformly \(^{13}\text{C}/^{15}\text{N}\)-labeled sample of a single base mutant of the oligonucleotide presented in Fig. 1 (nucleotide A1408G) in water at 5°. A single correlation is observed for each guanosine imino proton to its own H-8; previously one depended on through-space correlation in NOESY experiments to assign these protons.

Imino and amino protons often provide long-range NOEs. In RNA stems, NOEs from imino, amino, and adenosine H-2 protons are observed across the helix, unlike NOEs involving all other nonexchangeable protons.\(^{21}\) Furthermore, imino and amino protons in loops often make interesting contacts across loops; in the UUCG tetraloop, NOEs from the cytidine amino proton to sugar protons of the two tetraloop uridines indicate an amino hydrogen bond to a phosphate oxygen.\(^{22}\) Amino groups are often hydrogen bond donors in RNA-ligand recognition.\(^{23-25}\) The two amino protons can be hydrogen bond donors to the antibiotic and an RNA base simultaneously. Importantly, the cytidine and adenosine amino protons face the major groove, whereas those of guanosine face the minor groove (see later section).

Resonance Assignments of Antibiotic

Given its small size, resonance assignment of aminoglycosides would seem trivial compared to that of the RNA. However, there is significant

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FIG. 2. The NMR spectrum of an H(NC)-TOCSY-(C)H experiment on the A1408G mutant of the A-site oligonucleotide. This spectrum was acquired by the method of Simorre et al.\textsuperscript{16} on a 2.0 mM uniformly $^{13}$C/$^{15}$N labeled NMR sample in 5 mM sodium phosphate buffer, pH 6.2, at 5° on a Varian Inova 500-MHz spectrometer. The experiment accomplishes through-bond correlation of the guanosine imino H-8 protons. A peak is observed in the spectrum for each guanosine residue except G1408, which is not observed due to line broadening; the peak for the guanosine in the tetraloop (G15) is shifted outside this region of the spectrum. The total experimental time was 24 hr for 1024 complex $t_2$ points, 80 complex $t_1$ points, and 512 scans per FID.

overlap of proton resonances. Isotopic labeling, which is invaluable in assignment of the RNA molecule, is difficult to achieve for an antibiotic. Two possible methods of isotopic labeling exist: organic synthesis from a labeled small molecule precursor or isolation of the antibiotic from a producing organism after growth in uniformly labeled media. To date, neither method has been feasible in our laboratory. First, the antibiotics are too complicated to synthesize readily from a molecule such as glucose using chemical methods. Second, in our hands, the paromomycin producer, \textit{Streptomyces rimosus paromomycinus}, will produce antibiotic in rich, but not minimal, medium.

Without the aid of the heteronuclear correlation experiments applied
on isotopically labeled samples, assignments of NMR resonances for the antibiotic must be accomplished with homonuclear experiments and natural abundance $^1\text{H}/^{13}\text{C}$ correlation experiments. The primary experiments to apply are DQF-COSY, TOCSY, and $^{13}\text{C}^{-1}\text{H}$ HMQC. In the DQF-COSY, protons three bonds apart can be correlated to each other, allowing for resonance assignment by correlating protons attached to adjacent carbon atoms. This experiment uses the $^3J_{\text{HH}}$, which is dependent on the torsion angle between the two protons. The $^3J_{\text{HH}}$ varies between 12 and 1 Hz. Cross-peaks are only observed when the $J_{\text{HH}}$ is greater than approximately 1 Hz. The TOCSY experiment with a long mixing time (approximately 80 ms) complements the DQF-COSY with total correlation of all protons within a spin system. Dynamic averaging of the ring conformation leads to measurable coupling constants for all protons on adjacent carbons. An alternative to the DQF-COSY is a TOCSY experiment with a short mixing time (approximately 20 ms). The short mixing time allows for only three-bond correlation as in the DQF-COSY, while a longer mixing time allows magnetization transfer through the whole ring.

Assignment of the antibiotic is greatly aided by natural abundance $^{13}\text{C}^{-1}\text{H}$ HMQC. With sample concentrations of approximately 3 mM, single-bond proton–carbon correlations are obtained with a sufficient SNR in 8–12 hr on a modern NMR spectrometer. The HMQC experiment disperses proton resonances by carbon chemical shift, which is indicative of the type of proton. Aromatic carbon atoms such as those on the purine and pyrimidine bases of nucleic acids have downfield chemical shifts (100–160 ppm). The anomeric carbon of ribose and aminoglycoside sugars has the most downfield carbon chemical shift typically observed in the range of 85–100 ppm. Methyl carbon atoms are typically the most upfield shifted carbon atom, resonating in the 20–40 ppm range. The other carbon atoms in aminoglycoside antibiotics resonate between 40 and 90 ppm, similar to the C-2', C-3', C-4', and C-5' of RNA. Separation of the proton resonances by type of proton in an HMQC experiment eliminates ambiguities in assignment of the different types of protons that overlap in $^1\text{H}$ chemical shift. The exchangeable proton resonances are often not observed due to rapid exchange with solvent.

Forming RNA–Antibiotic Complex

**NMR Titration of Antibiotic into RNA**

Following completion of the assignment of the NMR resonances of each molecule free in solution, work can begin on the RNA–antibiotic complex. To form a complex, antibiotic is titrated into the RNA sample in water. The titration of the antibiotic into the RNA sample accomplishes three separate purposes. The first is to establish a drug–RNA complex suitable for further NMR experiments. The second purpose is to establish stoichiometry of the interaction, and the third purpose is to identify resonances that change on antibiotic binding.

The most downfield protons in the NMR spectrum, the NH (imino) protons of guanosine and uridine are easily followed by 1-D NMR, which makes them the most convenient marker for binding of the antibiotic to the RNA. Titration of the antibiotic paromomycin into an NMR sample of the A-site oligonucleotide (Fig. 3) yields an additional set of resonances,

![Fig. 3. Series of imino proton NMR spectra of the A-site RNA for free RNA, 0.5:1 paromomycin:RNA, and 1:1 paromomycin:RNA. Spectra were recorded at 25° in 10 mM sodium phosphate, pH 6.4, on a Varian Unity + 500-MHz spectrometer. Imino proton resonances that shift on addition of paromomycin are indicated, as are those of U1406 and U1495, which form a base pair.](image-url)
which results from imino protons on the RNA bound to the antibiotic. As antibiotic is added, the resonances from RNA in the free form disappear, and resonances in the bound form appear. At a stoichiometry of 1:1 RNA to antibiotic, the only resonances observed are those from RNA imino protons from RNA bound to antibiotic.

In the case presented in Fig. 3, the stoichiometry of the interaction was 1:1. However, multiple drugs could bind to one RNA molecule. For example, the titration of distamycin into a DNA oligonucleotide demonstrated that two drug molecules bind one DNA helix.30

The third purpose of a drug–RNA titration is to identify protons that change chemical shift on binding. In the paromomycin–oligonucleotide titration, two residues exhibit large chemical shift changes on antibiotic binding. This information enables one to map where the drug is binding on the RNA without any additional NMR experiments. Despite the low resolution of these data, they suggest further experiments and answer important questions such as whether different drugs induce equivalent or different effects on the RNA. Unfortunately, binding site mapping might be the only information that can be determined with NMR if the resonances in the complex are broadened. Broad resonances can make proton assignment and the identification of critical intermolecular NOEs much more difficult. The broadening of resonances primarily results from two phenomena: intermediate exchange of the complex on the NMR timescale and slower tumbling due to the larger size of the complex relative to the oligonucleotide alone.

**Affinity**

The affinity of the RNA-antibiotic interaction is the critical factor affecting what can be determined by NMR. A high-affinity interaction will likely be amenable to high-resolution structure determination of the complex, whereas a lower affinity interaction may be amenable to only low-resolution characterization. The effect of affinity on NMR data can be understood by analyzing the relationship between binding constants and chemical exchange.

The chemical reaction for the antibiotic binding to the oligonucleotide is

\[
RNA + drug \rightleftharpoons RNA \cdot drug
\]

For this reaction the binding constant of association \(K_A\) is defined as:

\[
K_A = \frac{[RNA \cdot drug]}{[RNA][drug]}
\]

where the [drug] is the concentration of free drug in solution and [RNA] is the concentration of RNA free in solution. The dissociation of the complex would be the inverse reaction, and thus the dissociation constant would be $1/K$. Thus, the expression for the dissociation constant, $K_D$ would be

$$K_D = \frac{[RNA][drug]}{[RNA \cdot drug]}$$

It follows that the $K_D$ would be equal to [drug] when 1/2 the sites on the oligonucleotide are occupied. A high-affinity interaction of RNA and drug would have a $K_D$ on the order of 1 $\mu M$. In the NMR tube with an initial RNA concentration of approximately 2 mM, the free RNA and free drug concentrations would be almost zero for a high-affinity complex. However, complexes that have a $K_D$ on the order of 2 mM would obviously be harder to study as half of the drug molecules would be free in solution at equilibrium.

The $K_D$ can be related to the rate of a simple one-step reaction. For the reaction:

$$RNA + drug \rightleftharpoons RNA \cdot drug$$

$$K_D = \frac{k_{-1}}{k_1}$$

where $k_1$ is the rate of the forward reaction and $k_{-1}$ is the rate of the reverse reaction.

In a diffusion-limited reaction, $k_1$ is approximately $10^7 M^{-1}s^{-1}$. Solving the equation for $k_{-1}$, using a value of 1 $\mu M$ for $K_D$, results in a rate constant for the reverse reaction of approximately 10 per second or a time constant on 0.1 sec. A weaker affinity complex of 100 $\mu M$ would therefore have a $k_{-1}$ of approximately 1000 per second or a time constant of 1 ms. These results can be related to chemical exchange observed by NMR and line-widths for NMR resonances of the complex.

**Chemical Exchange**

Three possibilities exist for the rate at which the complex dissociates as observed by NMR: slow exchange, intermediate exchange, and fast exchange. In slow exchange, the dissociation rate of the complex is much slower than the frequency difference between the free and bound resonances; in other words, the time constant or lifetime is much larger for the dissociation of the complex. For example, a $^1H$ chemical shift change of

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0.5 ppm for an RNA proton on binding to antibiotic on a 500-MHz (11.7 tesla) NMR spectrometer has a frequency difference of 250 Hz, or a time constant of 4 ms. A smaller chemical shift change of only 0.1 ppm would result in a time constant of 10 ms. As discussed earlier, the time constant for dissociation of a high-affinity (∼1 μM $K_D$) complex is approximately 100 ms. Thus, high-affinity binding results in slow exchange. Slow exchange results in the observation of two resonances at a substoichiometric ratio of antibiotic to RNA, one resulting from the free form and one from the bound form as observed in the titration shown in Fig. 3.

Intermediate exchange occurs when the dissociation rate is on the same timescale as the frequency difference of the two resonances. A time constant of 5–10 ms is approximately the timescale for the frequency difference between two resonances as determined earlier: 5 ms would translate into a $K_D$ of approximately 50–100 μM. This type of exchange results in broadening of the resonances at the binding site.

The third type of exchange is fast exchange where the dissociation rate of the antibiotic–RNA complex is fast on the NMR timescale, less than a millisecond, which results from weak binding (>100 μM). This type of exchange results in the observation of one resonance at a chemical shift between the resonance in the free form and in the bound form. The chemical shift of the resonance reflects the ratio of RNA bound to antibiotic to that of free RNA. The resonances affected by antibiotic binding continue to shift until all RNA molecules in the solution are saturated with antibiotic, which usually occurs at greater than 1:1 antibiotic to RNA stoichiometry.

Slow exchange is the optimal situation for NMR studies of an RNA–antibiotic complex. Typically, high-affinity RNA–aminoglycoside complexes on the ribosome have dissociation constants on the order of 1–5 μM, whereas those of aminoglycoside–RNA aptamers are often 2 orders of magnitude smaller (∼10 nM). NMR studies on a tobramycin–RNA aptamer complex ($K_D = 9$ nM) show the aminoglycoside binding tightly in the major groove of the RNA. High-affinity RNA aptamer–aminoglycoside complexes are in slow exchange making them amenable to NMR studies. Although it is possible to determine high-resolution structures of complexes not in slow exchange, it is much more difficult, so mapping the binding site may be the only realistic goal.

In cases of intermediate exchange, it is imperative that conditions of the NMR sample be changed to attempt to achieve slow exchange or fast exchange for the complex. NMR conditions that can be changed to escape intermediate exchange include salt concentration, pH, temperature, and

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even sample concentration. The broad resonances resulting from intermediate exchange are difficult to assign, and few NOEs may be observed, leading to few distance constraints for structure calculations. Nonetheless, broadening due to intermediate exchange can reveal resonances at the antibiotic binding site.

Mapping of Binding Site of Antibiotic on RNA

Chemical Shift

The 1-D NMR titration data suggest RNA residues that are in proximity to the antibiotic in the complex. Chemical shift is sensitive to local electronic environment. A positively charged drug binding to the RNA induces a significant change in the local environment of protons on the RNA and drug, which is reflected in different chemical shifts in the complex. Extensive analysis of chemical shift effects requires complete resonance assignment of both the RNA and antibiotic through application of the same NMR techniques discussed earlier.

With complete assignments, difference of chemical shifts in the free and bound form can be analyzed. Specific resonances that change on complex formation are grouped by nucleotide and by type of proton. The nucleotides whose resonances are most affected often constitute the binding site on the RNA. The type of proton that is affected indicates where the drug is binding on each nucleotide. If the drug binds in the major groove, changes in chemical shift for the easily identified pyrimidine H-5 and H-6 protons and purine H-8 protons are expected. If the drug binds in the minor groove, changes in chemical shift for adenosine H-2 protons and ribose H-1' protons are expected. The groups that face the minor and major grooves in A-form RNA are detailed in Table I.35

Whereas chemical shift is a sensitive method for analyzing the binding site, two problems exist for its exclusive use. First, conformational changes in the RNA structure on binding can also lead to changes in chemical shift for residues that are far from the binding site. A conformational change in the RNA on binding is an interesting result, but it is hard to distinguish by chemical shift alone between its effect and an effect of drug proximal to the binding site. Second, chemical shift is only low-resolution data. While theoreticians are trying to understand how to use chemical shift to determine tertiary structure, at this moment, chemical shift has not yet been successfully applied as a structural constraint for nucleic acids. Thus, more detailed analysis of the interaction must be applied to obtain a high-resolution structure.

Intermolecular NOEs

NOEs provide the most critical restraints for structure determination. Homonuclear and heteronuclear NOESY experiments applied to the RNA in the free form can also be applied to the complex. The determination of the structure of the RNA–antibiotic complex depends especially on intermolecular NOEs. Intramolecular NOEs can help define the structure of either of the individual components, but intermolecular NOEs define the conformation of the complex. Furthermore, the intermolecular restraints can act as long-range restraints for the RNA, actually improving the structure of the RNA oligonucleotide with the antibiotic acting as a rigid axis that fits into the oligonucleotide.

Intermolecular NOEs are distinguished from intramolecular NOEs by two methods. The first simply uses $^1$H chemical shift to demonstrate whether an NOE is intramolecular or intermolecular. RNA has defined regions where protons normally resonate (4–5 ppm for ribose protons, 5.1–6.2 ppm for anomeric protons and pyrimidine H-5 protons, 7–8.5 ppm for aromatic protons, 6–9 ppm for amino protons, and 10–14.5 for imino protons). Similarly, antibiotics have defined ranges where protons resonate; in the case of aminoglycosides, that range is primarily 2–5 ppm for all protons except the anomeric protons, which resonate between 5 and 6 ppm. Thus, any NOEs observed from a proton with a chemical shift upfield of 4 ppm to a proton downfield of 7 ppm would be expected to be an intermolecular NOE. In addition RNA has many more protons in the 5–6 ppm range, so an NOE observed from a proton in that region to one in the 2–4 ppm range could also be easily identified as an intermolecular NOE. These intermolecular NOEs are identified in a simple 2-D $^1$H–$^1$H NOESY. Thus, one can quickly identify the potential quality of a complex structure by scanning the NOESY spectrum of the complex in search of intermolecular NOES.
A second method for identification of intermolecular NOEs is through heteronuclear-filtered NOESY experiments. These experiments work by filtering out resonances resulting from either $^{13}$C or $^{12}$C (similarly $^{15}$N or $^{14}$N). Although the sensitivity of X-filtered experiments is less than that of $^1$H-only NOESY experiments, they can be invaluable in distinguishing between intermolecular and intramolecular NOEs. The X-filtered NOESYs work with a complex formed with uniformly $^{13}$C/$^{15}$N-labeled RNA bound to unlabeled drug; alternatively, the labeled drug bound to unlabeled RNA would also work, but labeled RNA is much easier to purify as discussed earlier. The NMR pulse sequence then filters out peaks resulting from the unwanted nucleus, so it is possible to have a spectrum with only NOEs from $^{13}$C-$^{13}$C (RNA-RNA), $^{13}$C-$^{12}$C (RNA-drug), or $^{12}$C-$^{12}$C (drug-drug). The intermolecular ($^{13}$C-$^{12}$C) NOEs are the critical constraints necessary for positioning the drug onto the RNA structure during structure calculations.

Structure Calculations

After intermolecular and intramolecular NOEs are assigned and torsion angles are determined, the structure of the complex is calculated using a simulated annealing protocol followed by restrained molecular dynamics and energy minimization. The calculation is performed in the same way as RNA in the free form. Many different computer programs have been applied to structure refinement based on NMR data through application of a simulated annealing protocol followed by restrained molecular dynamics and energy minimization. These include X-PLOR, CNS, and NMRArchitect (Molecular Simulations Incorporated, San Diego, CA). In this laboratory, NMRArchitect and X-PLOR have been applied.

The critical difference between structure calculations of the biomolecular complex and unimolecular systems is the ability to treat the two molecules separately and simultaneously. In that way, the structures of each individual component are not determined separately and then docked after refinement. Direct calculation of the complex structure is a more robust method for dealing with molecules with many degrees of freedom like the aminoglycoside antibiotics and RNA. NMRArchitect and X-PLOR both have that capability.

In X-PLOR, the two molecules need to be separately named with a file for each molecule defining the atoms connected by covalent bonds, and a file specifying the bond lengths and bond angles. These files are then converted into a structure file with all atoms having proper covalent geometry. The calculations then start from 100 structures with random torsion angles for both the RNA oligonucleotide and aminoglycoside. The random structures are subjected to a global fold protocol by first heating to 1000 K, while hydrogen bonding, distance, and dihedral constraints are gradually increased to full value over 40 ps of molecular dynamics. The RNA and drug are subsequently cooled to 300 K for 10 ps and energy minimized in the presence of Lennard–Jones potentials, but in the absence of electrostatics. Electrostatics are not used in the calculation because the $pK_a$ on each amino group is not known, and electrostatics may bias the calculated structure away from the observed NMR data. The total energies of the structures are analyzed, and the structures that converge to low energy are subjected to a structure refinement protocol. During refinement, the RNA–drug complex is heated to 1000 K for 30 ps of molecular dynamics with all hydrogen bonding, distance, and torsion angle constraints, and cooled to 300 K over 10 ps, again in the presence of Lennard–Jones potentials and without electrostatics. A final minimization step is performed in the same way. The resulting structures are finally superimposed to analyze the similarity of the structures.

Building on Structure

Comparison with Chemical Modification Data

Once the structure has been solved by NMR spectroscopy it can be compared to biochemical data. Chemical probing experiments have identified specific atoms that are modified in the absence of aminoglycoside and are protected from chemical modification in the presence of the antibiotic. Protected atoms should be buried in the structure, most likely at the RNA–

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antibiotic interface. Atoms that are modified in footprinting analysis of the complex would be exposed to solvent in the structure. Atoms could also be enhanced in modification, although no atoms in the antibiotic–oligonucleotide complex studied in this laboratory showed enhancement. These atoms should have greater solvent accessibility in the complex than in the free form.

Chemical probing experiments demonstrated that the model system mimics the aminoglycoside–rRNA interaction. If the structure does not agree with probing data, then the 3-D structure may be different under biochemical and NMR experimental conditions. Conditions ideally would be the same in both types of experiments. However, NMR requires sample concentrations on the order of 1 mM, whereas biochemical experiments require orders of magnitude lower concentrations of RNA (less than 1 μM). In addition, normal buffer conditions are different between the two sets of experiments, because biochemical experiments are often done at higher pH than those of NMR. Higher pH conditions in NMR experiments lead to faster solvent exchange of water-exchangeable protons and faster sample degradation over long time periods. Experimental conditions that mimic the biochemical experiments as closely as possible should then be tested by 1-D NMR and 2-D NOESY to determine whether the structure is truly different at different conditions. If experimental conditions have not led to different structures, then the data should be reanalyzed for possible discrepancies in interpretation.

Comparison with Mutational Data

The structure must be compared to results from mutational and modification-interference experiments. Important contacts defined in the structure should involve nucleotides that, when mutated or chemically modified, interfere with the binding of the antibiotic to the oligonucleotide. The antibiotic makes specific contacts to the conserved nucleotides of the internal loop. Nonspecific contacts are made to the phosphate backbone of nonconserved nucleotides in the lower stem.

Biochemical data indicated that specific nucleotides in the aminoglycoside binding site could be replaced by only one or two of the other three nucleotides with no effect on affinity. Ideally, the structure can explain these results. The location of a specific functional group on the base may be critical for binding of the antibiotic to the RNA. For example, the position 1495 of the A-site oligonucleotide (Fig. 1) can be G or U and still bind paromomycin with high affinity, since both sequences present a

carbonyl at this position.\textsuperscript{5} The structure of this complex shows that a carbonyl at this position forms a hydrogen bond to an amino group on the antibiotic, in agreement with the mutational result.\textsuperscript{48}

Testing Biological Predictions by NMR

If the biochemical and NMR data agree, then interesting features of the RNA–antibiotic complex are analyzed. In Fig. 4 (see color insert), two structures determined in this laboratory of the RNA oligonucleotide presented in Fig. 1B in its free form and complexed to the aminoglycoside antibiotic paromomycin are presented.\textsuperscript{48} In the 7-nucleotide asymmetric internal loop, three base pairs were identified: a U1406–U1495 pair, a Watson–Crick C1407–G1494 pair, and an A1408–A1493 pair. In addition, the N-1 atoms of A1492 and A1493 are pushed toward the minor groove, exposed to solvent, which is consistent with a model of hydrogen bonding of 2'-OH protons on the mRNA to A1492 and A1493.\textsuperscript{48} The antibiotic binds in the major groove, which is deep but narrow in RNA helices.\textsuperscript{35} This observation suggests a testable model of minor groove recognition of the mRNA by the rRNA since aminoglycosides cause misreading but do not competitively inhibit tRNA binding. While solution structures of RNA–antibiotic complexes can yield many interesting results, a structure of the complex is not the end of the project, because the structure will suggest new biochemical and structural experiments.

Use of Mutant Oligonucleotides

The 3-D structure of an RNA–antibiotic complex indicates which nucleotides on the oligonucleotide interact with specific positions on the drug. Mutations that should have no effect on binding of the antibiotic can be incorporated in the oligonucleotide. Chemical shift changes in the mutant RNA can be analyzed by 1-D titration and intermolecular NOEs can be analyzed by 2D NOESY and compared to the wild-type sequence. Mutations that affect binding are analyzed similarly by NMR. The intermolecular NOEs reveal whether the interaction with the antibiotic is disrupted only at the site of the mutation or throughout the RNA molecule.

Point mutants are often readily studied by NMR. A single base change is likely to affect only the chemical shift of resonances in the immediate vicinity of that nucleotide. In an RNA helix, nucleotides that are more than two base pairs away would not change in chemical shift unless the global structure of the mutant is different. Thus, the spectrum of the mutant

oligonucleotide is mostly unchanged from that of the wild-type oligonucleotide. Nonetheless, analysis of mutant RNA–drug complexes is often difficult. The $K_D$ for the aminoglycoside–RNA mutant complex is often higher, leading to intermediate chemical exchange rather than slow exchange as observed for the wild-type interaction. The NMR resonances become broader and harder to assign. Additionally, fewer intermolecular NOEs are observed for the weaker interaction, which makes high-resolution structure determination too difficult.

One biologically relevant mutation of the decoding site is A1408G. Position 1408 is not a universally conserved nucleotide. It is a guanosine in all eukaryotic ribosomes and an adenosine in all prokaryotic ribosomes. In the structure discussed earlier, A1408 forms a base pair with A1493. G1408 cannot replace A1408 in the same orientation and still form a base pair with A1493. Biochemical data demonstrated that this mutation weakens aminoglycoside binding affinity, as ring I sits in a binding pocket formed by the A1408 • A1493 base pair. Structure analysis of this mutant will further show how a guanosine at position 1408 can produce a functional ribosome and confer resistance to aminoglycosides.

Studies with Different Antibiotics

Although the various aminoglycosides contain some conserved chemical groups on rings I and II, variations both in the substituents on ring I and the total number of rings in the drug do exist. Studying the interaction of a variety of aminoglycosides with the oligonucleotide can reveal which of these groups are critical for the high-affinity binding of the drug to the RNA and if a common mode of interaction for the various aminoglycosides exists. Complexes of the RNA oligonucleotide with of a series of antibiotics similar to paromomycin have been studied. Neamine, which consists of only rings I and II of neomycin, binds specifically to the oligonucleotide and has antibacterial activity. It binds with lower affinity to the RNA, presumably because it lacks the additional contacts observed in the structure afforded by the presence of rings III and IV in neomycin and paromomycin. The lower affinity interaction of neamine with the oligonucleotide causes a more dynamic complex in fast exchange, making this complex harder to study by NMR. However, many of the chemical shift changes and intermolecular NOEs observed for the paromomycin complex are also observed for the neamine complex. Thus, neamine is still capable of recognizing

the critical nucleotides in the RNA sequence and inducing the important conformational changes.

This laboratory has determined 3-D structures of the oligonucleotide bound to either paromomycin or gentamicin as shown in Fig. 5 (see color insert).\textsuperscript{45,51} The primary difference between these two antibiotics is the linkage between rings II and III; ring III of gentamicin is linked to position 6 of ring II but this linkage is at position 5 in paromomycin.

The two structures determined by NMR show that rings I and II of the antibiotic interact with the RNA in a similar manner. A network of intermolecular hydrogen bonds lines the pocket in the major groove where the antibiotic recognizes the RNA oligonucleotide. The features on the RNA critical for recognition of antibiotic, including the U1406–U1495 base pair and the A1408–A1493 base pair, are observed in both structures. Ring III in gentamicin makes additional base specific contacts to G1405, which are not observed in the paromomycin–RNA complex. One of the most striking features is the similar conformational change observed between the free RNA and RNA bound with either paromomycin or gentamicin. On binding of either antibiotic, the N-1 of both A1492 and A1493 are displaced toward the minor groove. The structures of the two different classes of aminoglycosides have revealed how common chemical groups on the drug contribute to specific binding to the ribosomal target, and how the binding of either drug induces a similar conformational change in the RNA.

Drug Design

An obvious application of the RNA–antibiotic structures is structure-based drug design. Both the elements of the antibiotic that are critical for binding and the shape of the binding site on the RNA are known. Molecular modeling can be used to design novel drugs that contain the required chemical groups but which are attached to an alternate scaffold. Likewise, drugs could be designed to include the aminoglycoside scaffold but contain additional functional groups to increase specificity. These drugs can be made via organic synthesis and tested both for binding to the ribosomal target and antibiotic activity. Drugs that possess higher affinity and specificity for their ribosomal target can be refined and further tested. Although structure-based drug design does not always work, the potential for identifying new drugs in this manner is possible with the increasing number of structures of RNA–drug complexes and with the improving resolution of these structures.

Conclusion

Structure determination of RNA–antibiotic complexes by NMR provides a way to understand how an RNA sequence defines its structure, how a specific RNA structure relates to its biological function, and how a drug can be designed to fit into that structure. The methodology for RNA structure determination by NMR is now well established and can be applied to RNA–antibiotic complexes. Although an RNA free in solution can be quite dynamic, and sometimes difficult to study by NMR, high-affinity complexes of RNA oligonucleotide and antibiotic are less dynamic. With the vast improvement in NMR technology in the past few years, structures of RNA free in solution and in complexes with small ligands including aminoglycoside antibiotics will greatly increase in both number and quality.

Experimental Prerequisites for Determination of tRNA Binding to Ribosomes from Escherichia coli


Introduction

The tRNA binding features of 70S ribosomes from Escherichia coli have been extensively studied by several research groups. It has been established that these ribosomes contain three tRNA binding sites. The A site, which accepts the aminoacyl-tRNA during the first step of the elongation phase in protein biosynthesis, can also accept peptidyl-tRNA (or peptidyl-tRNA analogs); the P site, where the three possible forms of a tRNA, peptidyl-, aminoacyl-, or deacyl-tRNA can bind; and the E site, which shows a strong specificity for deacylated tRNA. The functional links between the three sites have been also established for the elongation phase of protein biosynthesis. The growing peptide chain is prolonged by one amino acid via three basic reactions. (1) the first reaction is the occupation of the A site by an aminoacyl-tRNA, which separates into a selection