Application of Residual Dipolar Coupling Measurements To Identify Conformational Changes in RNA Induced by Antibiotics

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Aminoglycoside antibiotics bind to 16S ribosomal RNA, inducing misreading of the genetic code and inhibiting translocation.[1,2] Aminoglycosides bind to short oligonucleotides that contain the ribosomal target; the oligonucleotides roughly mimic the affinity and specificity of the drugs for the ribosome.[3] Three-dimensional structures of several oligonucleotide−aminoglycoside complexes have shown that aminoglycosides induce a conformational change on the RNA sequence, supporting an induced fit model for aminoglycoside binding.[4−7] Understanding the structural requirements for specific drug binding requires determination of high resolution structures for many different drugs with different RNA sequences, but unfortunately that process requires the acquisition of numerous NMR experiments followed by structure calculations. Application of a single NMR experiment to test binding of the drug to the RNA and also the conformation of the RNA before and after complex formation would enable one to quickly identify the effect of the drug on the RNA structure.

Ligand−macromolecule interactions have been monitored by changes in proton, ^{13}C, or ^{15}N chemical shifts, which are sensitive to local environment changes caused by binding;[8−10] $^{1}H-^{15}N$ Heteronuclear Single Quantum Coherence (HSQC) spectra of proteins are ideal for studying binding in a protein−ligand complex because of the resonance dispersion, the short acquisition times required, and the spectral simplicity. Fesik and colleagues have used HSQC spectra to map ligand binding sites during drug screening.[11] In contrast, RNA does not have an equivalent NMR spectrum as indicative of conformation as the proton−amide nitrogen correlation that could be used for a drug screen. Additionally, binding of the drug to an RNA sequence does not necessarily mean the drug is active, since positively charged aminoglycosides will bind negatively charged nucleic acids nonspecifically.

Residual dipolar couplings have supplemented NOE and 3 bond *J*-coupling data to calculate high-resolution structures of proteins and protein−ligand complexes[12−16] and have been used to identify ligand geometry in protein−ligand complexes.[17,18] Dipolar couplings yield information on the orientation of proton−carbon and proton−nitrogen vectors with a molecular alignment axes, which complements short-range distance and torsion angle constraints.[19,20] Dipolar couplings are measured in a nondecoupled 2D single-bond correlation HSQC or HMQC in solution by first measuring the $^{1}J_{HC}$ of the RNA in standard conditions then remeasuring the $^{1}J_{HC}$ with the RNA suspended in a solution containing the filamentous phage PF1.[12,21] The phage particles align with the magnetic field and induce a partial alignment of the RNA. The scalar coupling is unaffected by the phage, thus, the dipolar coupling (*D*) can be determined from the two measurements: $D = {}^{1}J_{HC}(\text{aligned}) - {}^{1}J_{HC}(\text{not aligned})$. The dipolar couplings reflect orientation of individual bond vectors with respect to a fixed axes. If binding of aminoglycoside to the RNA induces a conformational change, the structural rearrangement should be observable by comparing dipolar couplings for the RNA in the free form and bound to antibiotic.

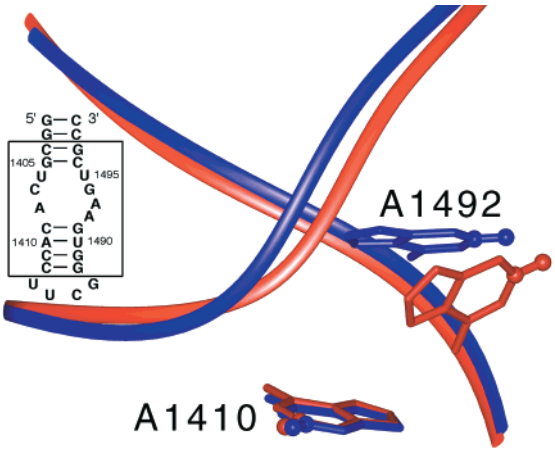

Figure 1. (A) Three-dimensional structures of decoding site oligonucleotide in its free form (blue) and bound to gentamicin C1A (red) determined by NMR.[4−6] The phosphodiester backbone for nucleotides U1406-A1410 and U1490-U1495 for both structures is shown as a solid oval. The bases of A1410 and A1492 are shown with the C2−H2 bond highlighted with a ball-and-stick representation. The secondary structure of the decoding site oligonucleotide is shown as an inset, above the structures. The numbering derives from that of *E. coli* 16S RNA.

The structures of the RNA oligonucleotide in its free form,[5] bound to paromomycin,[4] and bound to gentamicin C1A[6] have been previously determined. Base stacking through the asymmetric internal loop was observed for the RNA in its free form, including the four consecutive purines: G1491, A1492, A1493, and G1494. Upon complexation with either aminoglycoside, the two adenosines A1492 and A1493 are displaced toward the minor groove and are no longer stacked between G1491 and G1494, creating space for Ring I of both antibiotics to interact with the target RNA sequence. The conformational change of A1492 upon aminoglycoside binding is shown in Figure 1. Aminoglycoside

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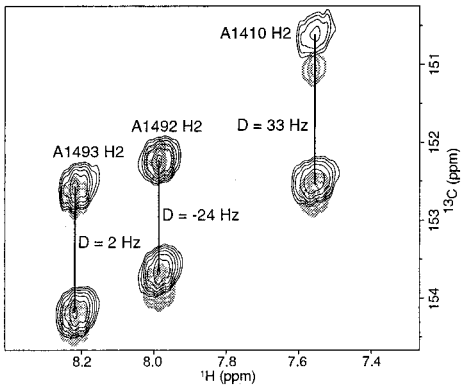

Figure 2. The adenosine C2−H2 portion of a 2D ω1-coupled sensitivity-enhanced ^{13}C/^{1}H HSQC acquired on the RNA oligonucleotide shown in Figure 1A bound to paromomycin is presented. The spectrum with thick gray contours is the RNA-drug complex in 10 mM sodium phosphate buffer, 10 μM EDTA, pH 6.4; the spectrum with thin black contours is the same complex after addition of the PF1 phage to ~21 mg/mL. The $^{1}J_{H2-C2}$ for each adenosine was measured to be 202 Hz ± 5 Hz. The dipolar coupling (*D*) value shown is determined by subtracting the measured splitting of the C2 carbon resonance by the H2 proton in the nonaligned spectrum from that in the aligned spectrum. The NMR spectra were acquired on a 500 MHz Varian Inova spectrometer at 25 °C; the spectra acquired were 64 scans of 2048 complex points in ω2 by 256 in ω1 with a ^{1}H sweep width of 5000 Hz and a ^{13}C sweep width of 3750 Hz. The data were zero-filled to 4096 points by 4096 points.

Table 1. Adenosine H2−C2 Dipolar Coupling Values (Hz)

	wild-type			A1408G	
nucleotide	free form	paromomycin	gentamicin	free form	paromomycin
A1408	31	18	26		
A1410	40	33	39	43	39
A1492	31	−25	−50	33	23
A1493	24	2	−6	15	15

binding induces an approximately 55° change in the angle between the A1492 C2−H2 bond and the rest of the molecule. A comparable change was observed with paromomycin (not shown).

Dipolar coupling measurements were applied to test whether this conformational change was observable by a rapid NMR method. A 2D ω1-coupled sensitivity-enhanced ^{13}C/^{1}H HSQC of the RNA oligonucleotide complexed to the antibiotic paromomycin without alignment and with partial alignment induced by the PF1 phage shows the appearance of residual dipolar couplings on alignment (Figure 2). The H2−C2 crosspeaks of A1410, in the lower stem, and A1492 and A1493, the internal loop residues displaced upon binding of antibiotic, are shown; the resonance for A1408 is outside of this spectral region. The chemical shift of each base proton of the entire molecule was measured before and after addition of the phage, and did not change significantly in line width or chemical shift, consistent with no change in the structure of the RNA induced by phage.

A comparison of dipolar couplings for the 4 adenosine H2/C2 resonances for the wild-type RNA oligonucleotide in its free form, bound to paromomycin, and bound to gentamicin C1A is presented in Table 1. In the calculated structures, A1410 in the lower stem is unaffected by binding of antibiotic and the measured dipolar coupling for the C2/H2 is consistently large and positive. Additional measurements (not shown) confirmed that the molecular alignment axes did not significantly change as a result of drug binding. A total of 57 dipolar couplings were measured for the free form oligonucleotide, 56 for the paromomycin bound oligonucleotide, and 36 for the gentamicin bound oligonucleotide (see Supporting Information). In the free RNA oligonucleotide and the RNA-drug complexes, the largest positive dipolar couplings were observed for the aromatic protons (H8−C8, H6−C6) in the lower stem (C1409−C1411, G1487−G1491); additionally, dipolar couplings for specific UUCG tetraloop resonances were always negative including the H1′−C1′, H2′−C2′, H3′−C3′, and H4′−C4′ of C14 and the H4′−C4′ of U13. The only significant changes occur at positions A1492 and A1493. This result confirms that qualitatively there is no change in alignment axes upon binding antibiotic.

A1408 changes orientation slightly with respect to the stem nucleotides upon binding of either antibiotic, agreeing with the dipolar coupling measurement. The structures of the two complexes and dipolar coupling values show that both A1492 and A1493 change conformation upon binding either paromomycin or gentamicin C1A. The larger change in the dipolar coupling of A1492 in comparison with A1493 is in agreement with the published structures, as A1492 exhibited a larger conformational change than A1493. The magnitude of the change for A1492 agrees with the range of calculated low energy structures and the error of the measurement.

The displacement of the A1492 and A1493 creates the binding pocket for Ring I of both classes of aminoglycosides allowing for high affinity binding of the drug to the RNA. The results presented in Table 1 show that residual dipolar coupling measurements can be used to identify the conformational change. The dipolar coupling measurements confirmed the conformational change of approximately 55° and 1.8 Å of A1492 and 35° and 3.2 Å of A1493 upon binding aminoglycoside.

An oligonucleotide containing position A1408G mutation was screened by residual dipolar coupling measurements to test for the conformational change in positions A1492 and A1493. *E. coli* grown with the A1408G mutant are resistant to many aminoglycosides,[22] and ribosomes with this mutant bind aminoglycosides with lower affinity.[23] In particular, paromomycin binds approximately 40-fold lower to the mutant ribosomes.[23] No large change in dipolar coupling for the A1492 and A1493 C2/H2 resonances was observed upon binding of paromomycin to G1408 mutant (right side of Table 1), in contrast to the results with wild-type oligonucleotide. The results show that no large conformational change takes place in the mutant sequence upon forming an antibiotic−RNA complex, consistent with the biological data.[22]

In summary, residual dipolar couplings can be used to test for specific conformational changes induced by drug binding to a target RNA sequence. The method is much more rapid than determining a drug/RNA complex structure by NMR. Non-decoupled HSQC spectra on 0.5 mM samples can be acquired in approximately 3 h on RNA−ligand complexes with size limitations of approximately 20 000 MW. An advantage of the dipolar couplings is the ability to correlate specific changes to changes in structure upon drug binding.

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Supporting Information Available: Table of dipolar coupling constants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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