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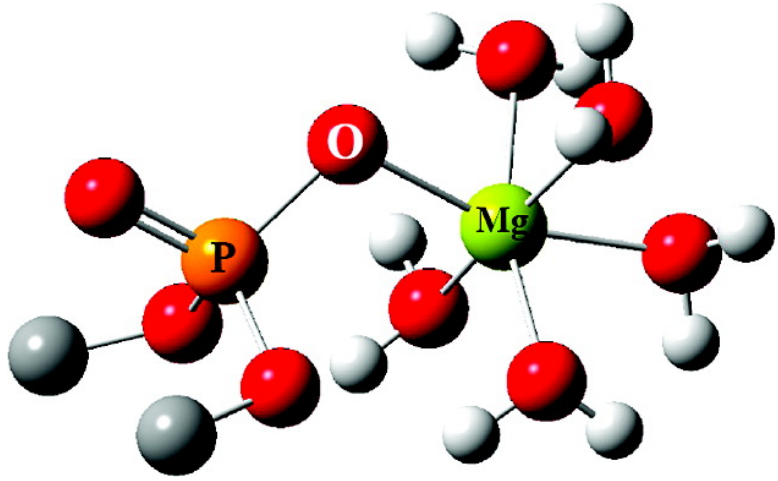

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Detection of Innersphere Interactions between Magnesium Hydrate and the Phosphate Backbone of the HDV Ribozyme Using Raman Crystallography

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Metal cation binding lies at the heart of much of RNA chemistry and is crucial for RNA folding and ribozyme catalysis.[1,2] Because RNA is a polyanion, metal−RNA interactions are both plentiful and complex. Current methods of characterization have limitations in their abilities to directly detect the type of metal−RNA interactions and their ligands. For example, while NMR spectroscopy can be used to look at binding and structures of outersphere complexes to RNA via $Co(NH_3)_6^{3+}$, evidence for innersphere sites is often less direct.[3] Phosphorothioate activity−rescue experiments, although providing a number of notable successes,[4] still require modification of the RNA and do not report on the coordination number of the metal ion.

A Raman microscope and Raman difference spectroscopy provide a complementary method to study the interactions of metal ions with complex RNAs. Here, we detect the vibrational signatures of RNA-bound magnesium hydrate in crystals of hepatitis delta virus (HDV) ribozyme and follow the effects of magnesium hydrate binding to the nonbridging phosphate oxygens in the phosphodiester backbone. Raman provides both a direct spectroscopic probe of innersphere contacts and characterization of the chemical nature of the complex. The Raman experimental approach used to elicit the data is described in Gong et al.,[5] and the HDV ribozyme was selected for our initial studies on magnesium binding on the basis of extensive biochemical and structural data.[6,7]

The HDV ribozyme is an ∼85 nt RNA that functions in the life cycle of the human HDV.[8] We developed a two-piece 71 nt construct for crystallographic studies in which the catalytic 2′-OH on the U-1 group was modified to prevent the cleavage reaction occurring in the crystals in the presence of Mg^{2+}.[5] The overall tertiary structure of HDV is shown in Figure 1, along with the metal binding sites. Herein, we demonstrate that in the HDV ribozyme crystals[9] there is a correlation between the Raman intensity of the signature peaks for innersphere magnesium hydrate (specifically Mg penta- and tetrahydrate) and the intensity of the PO_2^- symmetric stretch perturbed by magnesium binding, supporting a direct observation of $PO_2^-\cdots Mg^{2+}(H_2O)_x$ innersphere complexes (pentahydrate, $x = 5$; tetrahydrate, $x = 4$).

The properties of Mg^{2+} in aqueous solution, including its Raman and infrared active modes, have been well-characterized. The magnesium cation in solution exists as the stable hexahydrate[1] that has a totally symmetric stretching frequency at 360 cm^{-1},[10] which shifts to 341 cm^{-1} when the Mg^{2+} is surrounded by D_2O or $H_2{}^{18}O$ (confirmed in Figure S1, Supporting Information).

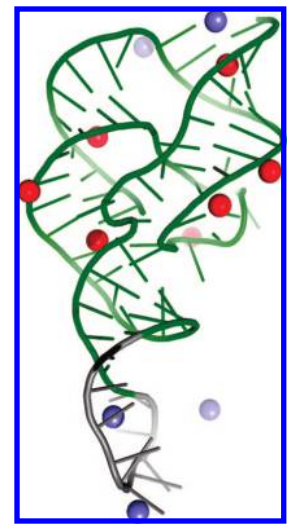

Figure 1. Crystal structure of the HDV ribozyme. The RNA is drawn with a coil representing the phosphodiester backbone and rods illustrating the position of the nucleotide bases. The native ribozyme sequence is green, and the non-native sequence introduced to bind the U1A protein is gray. Magnesium ions bound by the ribozyme core are red (see Table S1 and accompanying discussion), while those bound to non-native sequences or sequences not present in the RNA used in the current study are blue. Coordinates are from pdb entry 1cx0.

Using a Raman microscope, we recorded the Raman spectra of HDV ribozyme crystals in the presence of 0−40 mM Mg^{2+} in buffers containing 50−150 mM Na^+, which was present to maintain folding under low Mg^{2+} conditions. To obtain spectra in 0 mM Mg^{2+} conditions, magnesium was removed from the crystals by introducing 50 mM Na_2EDTA (raising [Na^+] to 150 mM) into the liquor surrounding the crystal. This process was repeated five times during 20 h to ensure that all bound Mg^{2+} ions have been removed from the crystal. Partial Raman difference spectra [HDV + 20 mM Mg^{2+}] minus [HDV no Mg^{2+}] are shown in Figure 2.[11] Signature features are observed near 1100−1120 and 322 cm^{-1} and can be attributed to phosphate groups on the RNA and to RNA-bound magnesium hydrate ions, respectively.

The differential feature near 1100 cm^{-1} is due to the symmetric stretch of PO_2^-.[12] In the parent Raman spectrum of the HDV ribozyme crystal in 20 mM Mg^{2+}, all 71 backbone $-PO_2^-$s in our construct contribute to a large peak near 1100 cm^{-1} (see Figure 4 in Gong et al.[5]). In the presence of Mg^{2+}, a fraction of the Raman signal for PO_2^- symmetric stretch (νPO_2^-) moves to 1117 cm^{-1}. The positive node in the differential (Figure 2) arises from

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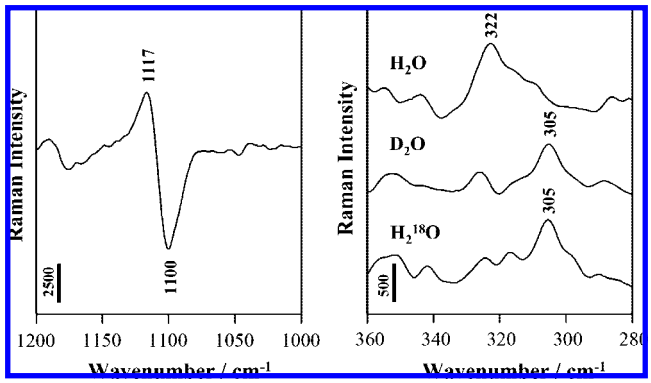

Figure 2. Raman difference spectra of HDV crystals [HDV +20 mM Mg^{2+}] minus [HDV no Mg^{2+}], pH 6.0, 647 nm, 60 mW laser excitation; 10 × 10 s data accumulation. Vertical bar represents photon events. Overall experimental details are given in Gong et al.[5] Left: partial Raman spectrum showing PO_2^- symmetric stretch of phosphate groups bound innersphere to Mg^{2+} at 1117 cm^{-1}; the negative differential at 1100 cm^{-1} is due to phosphate symmetric stretch of metal-free groups. Right: Raman signatures of Mg hydrate (pentahydrate and tetrahydrate are both possible) bound innersphere to PO_2^- oxygen.

magnesium-bound phosphate groups that form a new vibrational mode upshifted by ~17 cm^{-1} due to changes in vibrational coupling between the PO_2^- symmetric stretch and the adjacent C−O−P motions,[13] while the negative node at 1100 cm^{-1} results from the loss of a proportional fraction of the unbound or free PO_2^- symmetric stretch.

The second feature of note in the Raman difference spectra is the low intensity, but entirely reproducible, band near 322 cm^{-1}, which shifts to 305 cm^{-1} in D_2O or $H_2{}^{18}O$ (Figure 2). These isotope shifts identify the 322 cm^{-1} feature as a normal mode involving vibrational motions of the metal oxygen bonds (νMO) within magnesium hydrate. The likely identity of the magnesium hydrate species giving rise to the 322 cm^{-1} band can be deduced from the work of Peleg[14] who analyzed the properties of magnesium nitrate hexahydrate in aqueous solution and in ionic melts using Raman spectroscopy. In dilute aqueous solution, he identified the symmetric stretch of magnesium hexahydrate at 363 cm^{-1}, but in ionic melts where there are less than six water molecules per Mg^{2+}, a band at 322 cm^{-1} replaced this feature. The latter band was assigned to a magnesium hydrate mode where one or more of the coordinating waters are replaced by innersphere contacts to the nitrate oxygen. The assignment of a Raman feature in the 320−330 cm^{-1} region to innersphere-coordinated magnesium hydrate is further supported by a more recent study of Pye and Rudolf in aqueous $MgSO_4$.[10] Taken together, the isotope shifts and the literature studies lead us to assign the 322 cm^{-1} Mg hydrate band seen in the HDV ribozyme (Figure 2) to a magnesium complex that is six-coordinate but in which at least one of the ligands is an innersphere contact with a ligand from the RNA. This conclusion is further supported by quantum mechanical calculations carried out using Gaussian and by predictions of isotope effects (Supporting Information).

Magnesium hexahydrate in solution has perfect octahedral symmetry and has a symmetric stretching A_{1g} mode near 360 cm^{-1}, which moves to near 341 cm^{-1} upon D_2O or $H_2{}^{18}O$ substitution. This isotope shift is predicted by quantum mechanical calculations, although our calculations give 317 and 300 cm^{-1} for the water and isotopically substituted Mg^{2+} hexahydrate, respectively. One reason for the overshot is that the calculations used only single Mg^{2+} hexahydrate molecules, and these values are expected to increase as additional solvation shells are added.[10] Calculations for the Mg^{2+} pentahydrate model complex displayed in Figure 3A show

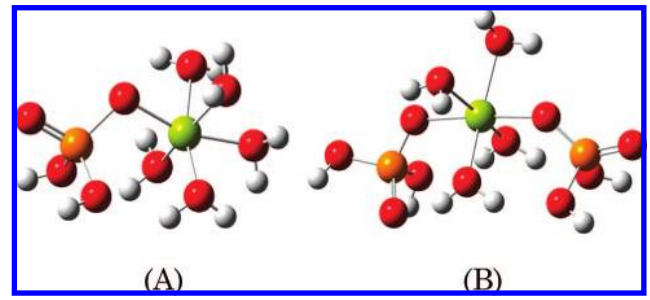

Figure 3. Models used in calculations. (A) Mg^{2+} pentahydrate−PO_2^- complex using $H_2PO_4^-$ as a model for the nonbridging $-PO_2^-$ group at an RNA site. (B) Mg^{2+} tetrahydrate−$2PO_2^-$ complex, with the two $-PO_2^-$ groups in the *trans* configuration. Colors of atoms: Mg^{2+} (yellow); oxygen (red); phosphorus (orange); hydrogen (gray). Calculations of analogues with dimethyl phosphate instead of $H_2PO_4^-$ show similar Mg^{2+} hydrate vibrational features.

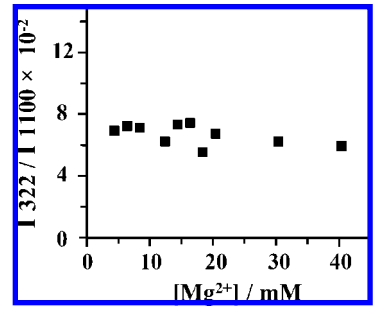

Figure 4. Ratio of intensities at 322 and 1100 (differential) cm^{-1} in the difference spectra as a function of Mg^{2+} concentration in liquor surrounding the HDV crystal.

that the νMO symmetric stretch is replaced by more complex motions involving the four equatorial waters and phosphate atom motions. This mode gives rise to a predicted feature at 295 cm^{-1}, which is a 22 cm^{-1} downshift as compared to the hexahydrate, consistent with the presence of pentahydrate in HDV. We also calculated two possible arrangements of magnesium tetrahydrate−$[PO_2^-]_2$ complex in vacuo, with two PO_2^- groups either at *trans* or *cis* positions in the octahedral template (Figure 3B). The results give values of 279 and 290 cm^{-1} for *cis* and *trans* magnesium tetrahydrate−PO_2^- complexes, respectively, and the latter value is very close to the calculated value of magnesium pentahydrate−PO_2^- complex. Thus, at the present level of our simple model compounds, it is difficult to use the calculations to confirm the exact values of the hydrate species in HDV. As such, both penta-Mg^{2+}-hydrate, tetra-Mg^{2+}-hydrate, or a mixture remain possibilities for the spectral features near 322 cm^{-1} in the HDV ribozyme.

Comparing the Raman signal for HDV in the presence and absence of Mg^{2+}, the appearance of the differential spectrum near 1100 cm^{-1} strongly suggests that $-PO_2^-$ groups are involved in innersphere contacts. This notion is confirmed by Mg^{2+} titration experiments. As the HDV crystals are exposed to increasing concentrations of Mg^{2+} in the surrounding liquor, the intensities of both the "1100 cm^{-1} negative differential" and the 322 cm^{-1} magnesium hydrate peak increase. As Figure 4 shows, there is a strong correlation between the two intensities, which strongly suggests that the appearance of the innersphere magnesium hydrate species is linked to the shift in the PO_2^- mode. Essentially identical spectra were obtained in the Raman spectra of aqueous solutions of the model compound dimethyl phosphate at molar concentrations of Mg^{2+} (Supporting Information, Figure S2). Dimethyl phosphate

should only be able to form 1:1 monodentate complexes with Mg^{2+}, thus demonstrating that a magnesium pentahydrate has a Ramen peak near the 322 cm^{-1}.

Using the 1100 cm^{-1} PO_2^- differential in Figure 2, we can estimate the number of phosphate groups per HDV molecule in innersphere complexes with $Mg^{2+}(H_2O)_x$s from the ratio of the attenuated Raman signal in the presence of Mg^{2+} (the area of the negative limb at 1100 cm^{-1} in the difference spectrum) to that of the 1100 cm^{-1} peak in the parent spectrum of the HDV crystal in the absence of Mg^{2+}. At 20 mM Mg^{2+}, this ratio is ~0.07, and since there are 71 PO_2^-s in the HDV molecule, this suggests that there are on average ~5 phosphates bound to $Mg^{2+}(H_2O)_x$s under these conditions. While the above ratio changes as a function of Mg^{2+} concentration, a titration from 4 to 40 mM Mg^{2+} suggests that the estimated value of ~5 metal bound phosphates reflects a plateau in the dependence of the above ratio on Mg^{2+} concentration (Figures 4 and S3). This estimated number of phosphates bound is necessarily a lower limit because Raman signal may reflect partial occupancy of a larger number of sites, and because of the nature of the differential, some intensity may be lost when the two parent peaks are close together.

The presence of five innersphere $-PO_2^-\cdots Mg^{2+}$ contacts is consistent with the published X-ray structure for the wild-type product form of HDV,[6] which reveals five possible innersphere contacts ($O^-\cdots Mg^{2+}$ distance $\leq$ 2.8 Å)[15] between Mg^{2+} and $-PO_2^-$ and two contacts with N_7 and O_6 atoms on a guanosine residue (see Supporting Information for details). It is also consistent with absorbance-detected Mg^{2+} titrations in solution on the cleaved form of the ribozyme that showed a change in absorbance over similar range of 10−100 mM Mg^{2+}.[16] Unfortunately, it is problematic to make direct comparison between the binding data in solution and in the crystal given herein since Na^+ concentrations in the solution experiments were 20-fold higher. Similarly, the RNAs used in the current study differ slightly in sequence from those in previous studies, though it is unlikely that such sequence differences would lead to significant changes in the overall HDV structure or in the number of metal ions bound.

The close agreement in the predicted number of metal-bound phosphates between Raman, X-ray, and other solution studies, however, suggests characteristic features of the Raman signal itself that allow it to be used as a tool to study metal ion binding. Specifically, while the metal-induced attenuation and upshift in the Raman signal for $-PO_2^-$ represents the combined signal of Mg^{2+}−phosphate interactions from all $-PO_2^-$ positions in the HDV molecule, it is also true that the relative contribution of individual $-PO_2^-$ positions to metal-induced changes in the Raman signal will reflect the extent to which they are bound by Mg^{2+}. Within a complex folded RNA, the relative occupancy of Mg^{2+} at $-PO_2^-$ positions involved in specific metal ion coordination is expected to be significantly larger than that predicted for nonspecific interactions with $-PO_2^-$ positions in other regions of the molecule. The crystallographic studies noted above show that HDV is no exception. Thus, the observation that metal-induced changes in the Raman signal for $-PO_2^-$ in HDV is both saturable (Figure S3) and predicts the same number of metal ions identified by an independent method suggests that the contribution of nonspecific metal interactions to changes in the $-PO_2^-$ Raman signal of HDV is likely to be small relative to those predicted to be involved in direct innersphere coordination. The ratio of the attenuated $-PO_2^-$ Raman signal in the presence of Mg^{2+} to that of the parent spectrum of $-PO_2^-$ in the absence of Mg^{2+}, therefore, appears to provide an accurate estimate of the number of metal ions involved in direct innersphere interactions.

In summary, we have shown that Raman crystallography can provide a powerful approach for studying metal ion interactions in RNA that can complement traditional but less direct crystallographic and biochemical methods. This approach permits both qualitative and semiquantitative dissection of metal ion binding and should prove useful in the analysis of other functional RNA and RNA−protein complexes. Raman experiments also provide the potential to detect site-specific effects using isotope and chemical substitutions,[17] indicating broad potential of this approach.

Acknowledgment. We thank Drs. V. Anderson and M. Harris for discussions on dimethyl phosphate.

Supporting Information Available: Raman spectrum of aqueous $[Mg(H_2O)_6]^{2+}$, quantum mechanical calculations, interaction between magnesium hydrate and dimethyl phosphate in aqueous solution, and Mg^{2+} binding sites in the crystal structure of HDV. This material is available free of charge via the Internet at http://pubs.acs.org.

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