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Published on Web 03/14/2002

Sequestering of Eu(III) by a GAAA RNA Tetraloop

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Received October 1, 2001

Abstract: The site-specific binding of metal ions maintains an important role in the structure, thermal stability, and function of folded RNA structures. RNA tetraloops of the "GNRA" family (where N = any base and R = any purine), which owe their unusual stability to base stacking and an extensive hydrogen bonding network, have been observed to bind metal ions having different chemical and geometric properties. We have used laser-induced lanthanide luminescence and isothermal titration calorimetry (ITC) to examine the metal-binding properties of an RNA stem loop of the GNRA family. Previous research has shown that a single Eu(III) ion binds the stem loop fragment in a highly dehydrated site with a K_d of ~12 μM. Curve-fitting analysis of the broad luminescence excitation spectrum of Eu(III) upon complexation with the tetraloop fragment indicates the possibility of two microenvironments that do not differ in hydration number. Binding of Eu(III) to the loop was accompanied by positive enthalpic changes, consistent with energetic cost of removal of water molecules and suggesting that the binding is entropically driven. By comparison, binding of Mg(II) or Mn(II) to the RNA loop, or Eu(III) to the DNA analogue of the loop, was associated with exothermic changes, consistent with predominantly outer-sphere coordination. These results suggest specific binding, most probably involving ligands on the 5′ side of the loop.

Comparative sequence analysis of large RNAs has revealed that tetraloops (terminal loops containing four nucleotides) of the GNRA (RNA stem-loop sequence of four residues, where G = guanosine, N = any base, R = any purine, and A = adenosine) and UNCG families, occur with disproportionate frequency over larger loops predicted to be favored thermodynamically.[1] Solution NMR studies have shown that the basis of the unexpected stability is a combination of base stacking and a hydrogen bond network involving ribose 2′OH and backbone phosphate oxygen atoms.[2,3] In addition, hydrogen bond donors and acceptors are available to form RNA−RNA[4,5] and RNA−protein [6−8] interactions in biological systems. A thorough understanding of the thermodynamics, stability, and ligand binding properties of these tetraloops is therefore an important issue in the structural biology of RNA.

Although the first solution structure of a GNRA tetraloop was solved by Heus and Pardi in the absence of multivalent metal ion,[2] Brownian dynamics calculations by Hermann and Westhof predicted a likely binding site for divalent metal ion in a GNRA motif as part of the sarcin-ricin loop,[9] and a number of different ions have since been shown to bind with various affinities. Rüddisser and Tinoco have shown that both Co(III) hexammine, a mimic of fully hydrated Mg(II) ions, and Mg(II) bind the loop sequence weakly, and selective line broadening by Mn(II) provided evidence that it binds as well.[10] The observation that Mg(II) binds more tightly than Co(III) hexammine was interpreted as a preference for at least one inner-sphere coordination to the metal ion. ^{31}P NMR studies of Mg(II) and Cd(II) binding to a GAAA loop sequence by DeRose and colleagues implicated the phosphate oxygen atoms of the stacked 3′ side of the loop in the binding.[11]

Similarities between trivalent lanthanide ions and alkaline earth metal ions in terms of metric properties and ligand preferences, as well as their favorable spectroscopic characteristics,[12,13] have advanced the use of trivalent europium (Eu(III)) ions as probes of complex formation with biological macromolecules.[14] The approach has provided specific information about individual binding sites in small organic molecules.[15,16] Both the ground state ($^7F_0$) and the excited state ($^5D_0$) of Eu(III) are nondegenerate, so each distinct Eu(III) environment exhibits a singlet peak in the excitation spectrum. Because the $^5D_0 \rightarrow {}^7F_2$ transition at 614 nm is hypersensitive, emission is enhanced

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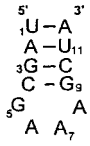

Figure 1. Secondary structure of the RNA stem loop fragment used in these studies. The numbering scheme shown here and used in the text is specific to this particular construct.

for the metal−ligand complex relative to that of unbound metal ion in aqueous solution.

By exploiting the changes in luminescence lifetime, intensity, and excitation wavelength of lanthanide ions that occur upon specific binding to RNA, we have recently shown that Eu(III) binds to a tetraloop with the loop sequence GAAA with moderate affinity, and that this site can be competed by an excess of Mg(II).[17] We also found that the Eu(III) is coordinated to only 1−2 water molecules (out of ≤9 possible sites), suggesting that there may be as many as 7−8 inner-sphere coordinations to the RNA. The blue shift in the excitation spectrum of Eu(III) (relative to the excitation peak of the ion in water) upon binding to the RNA loop is consistent with the metal ion residing in a coordination environment characterized by lesser energy stabilization than that occurring upon coordination with water.[18,19] These data led us to conclude that Eu(III) was bound in a highly specific, dehydrated "cage".[20] On the other hand, the observed binding of a variety of metal ions having different properties argues that tetraloops, despite their small size, provide a heterogeneous set of molecular environments. The broadness and asymmetry of the blue-shifted excitation peak also prompted us to question whether the heterogeneity extends to the binding properties of a single ion, Eu(III).

We have therefore undertaken a deeper understanding of the intricate metal binding properties of the GAAA tetraloop hairpin as well as the energetics driving the tetraloop−metal ion interaction. Further analysis of luminescence excitation spectra of Eu(III) ions bound to the GAAA hairpin loop supports the heterogeneous nature of Eu(III) binding. Calorimetric measurements indicate that the binding of Eu(III) to the RNA tetraloop is highly endothermic, consistent with removal of water molecules. In contrast, binding of the cations Mg(II), Mn(II), and Co(III) hexammine to the RNA fragment, as well as Eu(III) to the less structured DNA analogue, was exothermic, implying predominantly water-mediated interaction. These data support a model of Eu(III) being encapsulated by multiple coordinations, most likely belonging to RNA ligands on the unstacked 5′ side of the loop.

Experimental Section

A 12-nucleotide stem loop sequence including a "GNRA" tetraloop sequence (5′UAGCGAAAGCUA3′, where the underlined bases represent those located in the loop; Figure 1) and its DNA analogue were synthesized by standard phosphoramidite chemistry on an Applied Biosynthesis peptide system (ABi) converted for nucleic acid systems.[21] An advantage of the synthesized samples is that they have hydroxyl groups at both termini, thereby minimizing the likelihood of nonspecific metal binding to the free 5′ phosphate generally found on transcribed oligomers. The deprotected oligomers were purified by standard HPLC methods and concentration of strands was determined from their absorbance at 260 nm using an extinction coefficient ϵ_{260} of $1.28 \times 10^5\ M^{-1}\ cm^{-1}$. RNA molecules were folded by heating to approximately 90 °C in 10 mM MES (2-(morpholino)ethanesulfonic acid monohydrate) buffer (pH 5.5) and cooled gradually before the addition of NaCl to a final concentration of 100 mM. The integrity of RNA and DNA oligomers was verified by denaturing gel electrophoresis. The presence of a predominant conformer was ascertained by nondenaturing gel electrophoresis in which the acrylamide gel mixture included 100 mM NaCl, the same as in the assay conditions used for luminescence and ITC (isothermal titration calorimetry) experiments. On the basis of the relative mobility of the folded RNA tetraloop fragment with respect to duplex RNA markers, we concluded that the species present was the hairpin monomer. Identity of the folded molecule as the hairpin was verified by comparison of NMR spectral features with previously published chemical shifts and NOE cross-peaks.[2]

Stock solutions of Eu(III) were prepared by methods detailed in the literature[22] and were kept at a concentration of not more than 0.1 M, and at a pH between 2 and 3, to discourage base hydrolysis encountered at high concentrations and high pH.

Luminescence Measurements. Luminescence studies were performed as detailed previously.[17] Eu(III) in solution (nominal volumes of 300 μL) was excited directly with a pulsed dye laser and luminescence intensity and lifetime were documented and analyzed with respect to experimental conditions. The $^7F_0 \rightarrow {}^5D_0$ excitation spectra of Eu(III) were acquired by scanning the dye laser at the rate of 1 cm^{-1}/step over the 0−0 transition (17230−17320 cm^{-1}, equivalent to 580.4−577.4 nm). The sample emission from the $^5D_0 \rightarrow {}^7F_2$ hypersensitive transition was monitored at 614 nm.

Luminescence decay lifetimes were determined by positioning the laser at the desired excitation maximum and recording the Eu(III) emission at 614 nm. Unless otherwise stated, an acquisition gate of approximately 0.26−0.28 ms (relative to the laser trigger pulse) was chosen to minimize spectral contribution of fully hydrated (i.e. unbound) metal ion to the total excitation.

Curve Fitting and Regression Analysis. The Marquardt nonlinear regression method[23,24] was used to resolve components of the Eu(III) excitation spectra, and data were analyzed by GRAMS 386 software. Three parameters used to define a peak were the wavelength of peak maximum, maximum intensity, and the formula width at half-maximum height (fwhm). The curve fitting protocol involves initial estimates of the parameters and a systematic variation of parameters until solutions converge. A comprehensive description of the method has been well documented.[25]

Calorimetric Analysis. Isothermal titration calorimetry (ITC) studies were conducted on a Microcal VP-ITC ultra-sensitive isothermal titration microcalorimeter. The VP-ITC unit, with a precise temperature control set at 25.0 ± 0.1 °C, directly measured heat evolved or absorbed in liquid samples as a result of injection of reactant.[26] The reference cell was filled with Millipore ultrapure deionized water. All samples were extensively dialyzed in 10 mM MES buffer of pH 5.5 with 100 mM NaCl added as counterion. Samples were filtered and degassed by gently stirring the sample under vacuum for 8 min using the Thermo-

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vac (a vacuum thermostating system) prior to introduction to the calorimeter. Each of 40 injections of 6 μL of a metal ion solution (from stock solutions of 0.5−50 mM) was delivered into a solution of RNA (or DNA) over 12 s by a syringe spinning at 270 rpm, with a 240 s equilibration time between injections. The instrument was controlled by Microcal Observer software comprising a 16-bit A/D converter board for data acquisition and a second interface board for calorimetric control. Data from control experiments in which metal ion was injected into buffer were subtracted from all RNA data to correct for heats of dilution. Using a previously determined stoichiometry of one Eu(III) ion per tetraloop,[17] a simple binding model described by a ligand binding to a single set of n identical, noninteracting sites on a macromolecule[26] was used to obtain the binding constant, enthalpic (ΔH), and entropic (ΔS) terms contributing to the Gibbs free energy (ΔG) of association.

Results

Deconvolution of the Excitation Spectrum of the Eu(III)−RNA Complex. We have previously shown that direct excitation of Eu(III) bound to a GAAA tetraloop hairpin (sequence in Figure 1A) resulted in an excitation spectrum of the $^7F_0 \rightarrow {}^5D_0$ transition that was broad, asymmetric, and blue shifted by 20−30 cm^{-1} with respect to the 17273 cm^{-1} (578.9 nm) peak for the Eu(III) aqua-complex.[17] From the increase in luminescence intensity upon complex formation, we calculated a stoichiometry of one Eu(III) ion per tetraloop fragment and a dissociation constant of ∼12 μM. The first objective in this study was to analyze the excitation behavior with the goal of deconvoluting the spectrum into individual components.

Both the ground and excited state of Eu(III) are nondegenerate, so each distinct binding environment exhibits a singlet peak in the excitation spectrum.[12,15,27] The 4f electrons are shielded from the ligand electron density by the presence of electrons in the outer fifth shell, resulting in only minor perturbations of electronic transitions between the energy levels of these f orbitals. The minor energy changes experienced by the f electrons resulting from different ligands or coordination states of the lanthanide ions are reflected in the detailed shape of the emission patterns observed from the excited state. Consequently, these energy changes can provide a valuable approach to determining the nature of the chelation experienced by the ions.[20] Eu(III) complexes having homogeneous binding sites are characterized by excitation peaks that are sharp and narrow (fwhm ∼15 cm^{-1}).[28] The broad, asymmetric excitation spectrum obtained for the Eu(III)−RNA tetraloop complex therefore suggests the summation of several contributory peaks (Figure 2A). The spectral line shapes of the absorption and emission transitions of lanthanide ions, such as Eu(III) in aqueous solution, have generally been considered to be Lorentzian in character. Upon binding to slightly different environments, the Lorentzian character is distorted resulting in a spectrum described by a mixture/hybrid of the Lorentzian and Gaussian functions.[29] Our data therefore were fitted to a Lorentzian−Gaussian profile.

Resolving the excitation spectrum into its respective components is essential to understanding the nature of the binding site. Excitation spectra from six independent data sets were decomposed into component peaks having Lorentzian−Gaussian line shape.[25] The spectra were best fit by the sum of two distinct major peaks centered at 17286 and 17303 cm^{-1} (Figure 2A). When [Eu(III)] was in excess of [RNA], an additional and independent peak was observed at 17273 cm^{-1}, and was attributed to the fully hydrated Eu(III) ion. Individual data sets had additional small peaks (<5% of the area of the larger two peaks) that were not reproducible, and were considered more likely to represent noise than a distinct environment. The excitation spectrum at low [Eu(III)] ([Eu(III)]:[RNA] ≪ 1) was fitted almost completely by the 17286 cm^{-1} peak, suggesting a preference for that site. Titration of Mg(II) or Mn(II) into the Eu(III)−RNA mixture resulted in diminution of both peaks without any consistent preference for either (Figure 2, B and C).

Because the intensity of an excitation peak is affected by factors such as the absorptivity coefficient of the ion in that site and its quantum yield, the relative peak heights may not correlate directly with the occupation of the various sites. The relative intensity of a given peak, however, is proportional to the occupation of that particular site by Eu(III). These results imply that Eu(III) is bound to two distinct sites or to a heterogeneous site where Eu(III) coordination to ligands (including water) varies.

Competition of the Eu(III) Site with Other Cations. One way to ascertain whether two metal ions bind to the same or overlapping sites is to measure competition for an observable feature. We had previously shown that a 150- to 450-fold excess of Mg(II) decreased the Eu(III) luminescence by 50%, consistent with (but not unambiguous proof of) a scenario in which the two metal ions compete for the same site. To ascertain the binding characteristics of metals such as Mn(II) and Co(III) hexammine, we now extended these experiments to competition with other ions. Competition experiments performed similar to that with Mg(II) indicated that Mn(II) has at least 300-fold lesser affinity, i.e., $K_d \geq 3$ mM. Addition of >100-fold excess of Co(III) hexammine resulted in no observable change in the excitation spectrum of the Eu(III)−RNA complex.

Hydration Properties of the Two Sites. To evaluate the possibility that the heterogeneity in the sites revealed by deconvolution of the broad excitation peak is due to different hydration numbers, we measured the number of water molecules coordinated to Eu(III) in each milieu. The number of water molecules bound to the inner sphere of the ion, and hence the number of direct vs water-mediated (outer sphere) coordinations between ion and ligand, can be examined on the basis of their differing excited-state lifetimes.[30] We exploited this property to characterize the lifetimes corresponding to the different peaks. A single Eu(III) ion environment displays a monoexponential decay, whereas if there is variation in the number of bound water molecules or a substantial difference in the relative quenching by different ligands, a multiexponential curve may result. Analysis of the luminescence decay upon excitation at the center of the broad peak at 17290 cm^{-1} yielded an apparently monoexponential curve with a decay constant, k_{obs}, of 1.78 ms^{-1}, consistent with a single environment. Using the empirical equation derived by Horrocks:[30]

$$Q_{Ln} = A_{Ln}(k_{obs} - k_{D_2O}) \quad (1)$$

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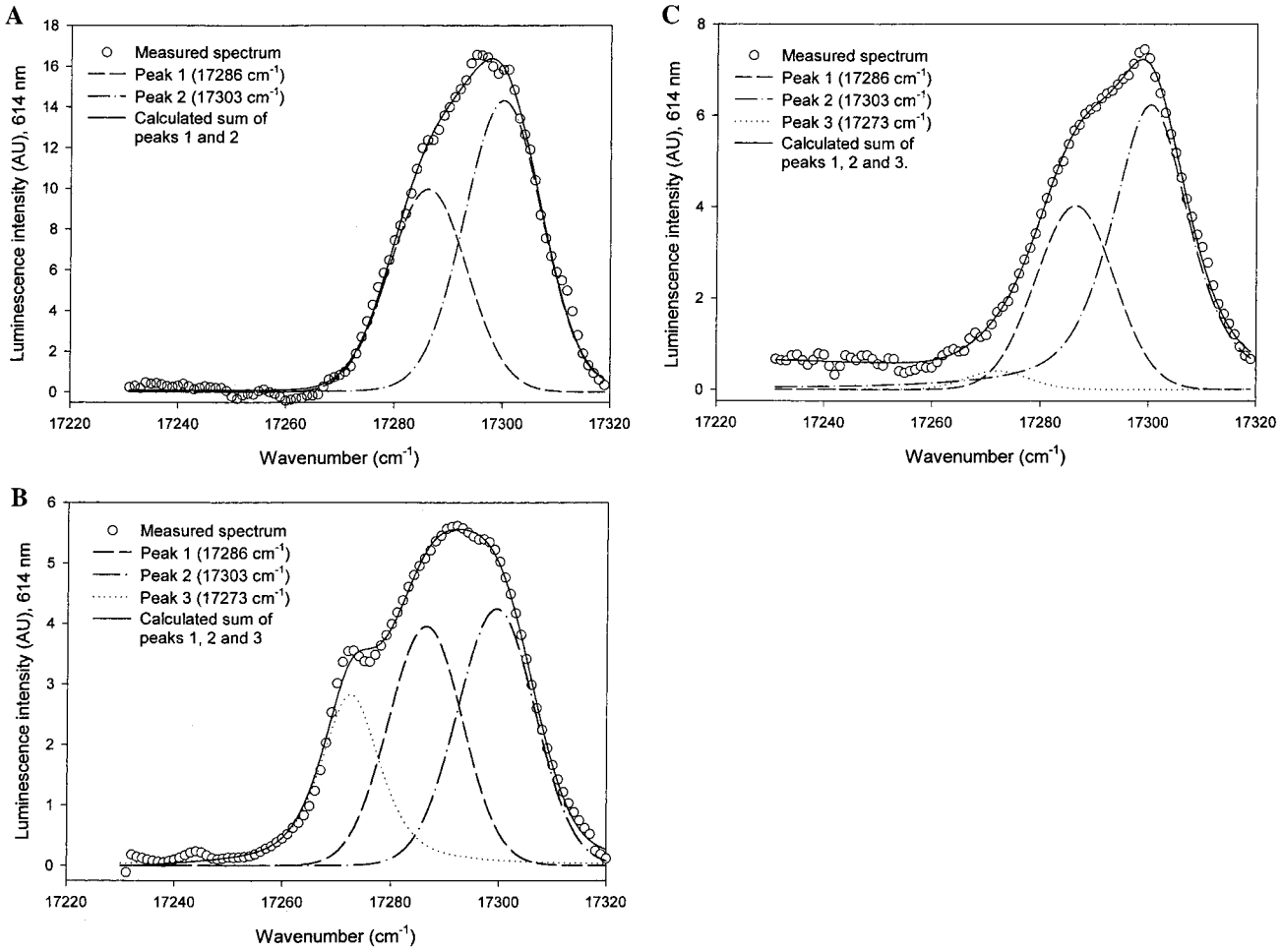

Figure 2. The measured $^7F_0 \rightarrow {}^5D_0$ excitation spectrum, resolved components, and calculated sum spectra of the components from solutions of the following composition: (A) 40 μM Eu(III) and 35 μM GAAA RNA tetraloop hairpin and 100 mM NaCl in 10 mM MES buffer, pH 5.5. The spectrum was resolved into two Lorentzian−Gaussian peaks with the following parameters: Peak 1 ($\lambda_{1max} = 17286$ cm^{-1}, fwhm (σ_1) = 16 cm^{-1}); Peak 2 ($\lambda_{2max} = 17303$ cm^{-1}, $\sigma_2 = 17$ cm^{-1}), where λ is the wavelength of the peak maximum, and σ is the full width at half-maximum intensity. (B) Effect of added 1.5 mM Mg(II) on the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of the sample used in part A. Deconvolution of the excitation spectrum was performed using the same parameters as in part A, with the addition of a peak corresponding to fully hydrated Eu(III) at $\lambda_{max} = 17273$ cm^{-1}, $\sigma_2 = 15$ cm^{-1} (λ and σ are defined in the legend of Figure 1). (C) Effect of added 2.0 mM Mn(II) on the $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of the same sample shown in part A. Deconvolution of the excitation spectrum was performed using the same parameters as in part B.

where Q_{Ln} is the number of water molecules directly coordinated to the lanthanide ion, and k_{obs} and k_{D2O} are the observed luminescence decay constants for the complex in aqueous solvent and D_2O in ms^{-1}, respectively. For Eu(III), the constant $A = 1.05$, and k_{D2O} was measured as 0.40 ± 0.02 ms^{-1}.[17] The k_{obs} measured for the complex thus correlates with 1−2 bound water molecules (out of a possible nine) and therefore up to 7−8 direct coordinations to the RNA. Although small quenching effects of ligands other than water have been neglected in this calculation, they may contribute to the noninteger value of Q_{Ln} and an underestimate of the number of nonwater ligands.

We have previously been successful in deconvoluting biexponential decay curves of Eu(III)−RNA complexes in the presence of excess Eu(III) into two components corresponding to those of the complex and the fully hydrated Eu(III).[30] Although the decay resulting from excitation at 17290 cm^{-1} (which includes contributions of each of the two peaks observed on deconvolution) was best fit by a single monoexponential curve, we did not want to exclude the possibility that each peak represented a binding site with a different hydration number. We therefore measured decay rates after exciting at each of the individual peak maxima, where there was very little overlap. Excitation of the peaks at 17286 cm^{-1} and at 17303 cm^{-1} resulted in monoexponential decay curves ($R^2 > 0.98$) with decay rates of 1.70 ± 0.06 and 1.82 ± 0.02 ms^{-1}, respectively. According to these data, the mean number of bound water molecules, 1.37 and 1.49 (±0.5), bound to each site was essentially the same within experimental error, and implies that the two sites are distinguished by some criterion other than hydration.

Binding of Eu(III) to the DNA Analogue of the GNRA Tetraloop. The binding of Eu(III) to the DNA analogue of the GAAA tetraloop fragment, which is known to be less structured than the RNA sequence,[31,32] was also measured. In contrast with the blue-shifted excitation of Eu(III) complexed with the RNA loop, direct excitation of Eu(III) bound to the DNA analogue resulted in a spectrum with a peak maximum centered at 17273 cm^{-1}, i.e., unchanged from the location of the Eu(III) aqua-complex, and displayed no significant line broadening. When excitation spectra were obtained at the same acquisition gate as was used for the RNA−Eu(III) data (0.26−0.28 ms), no

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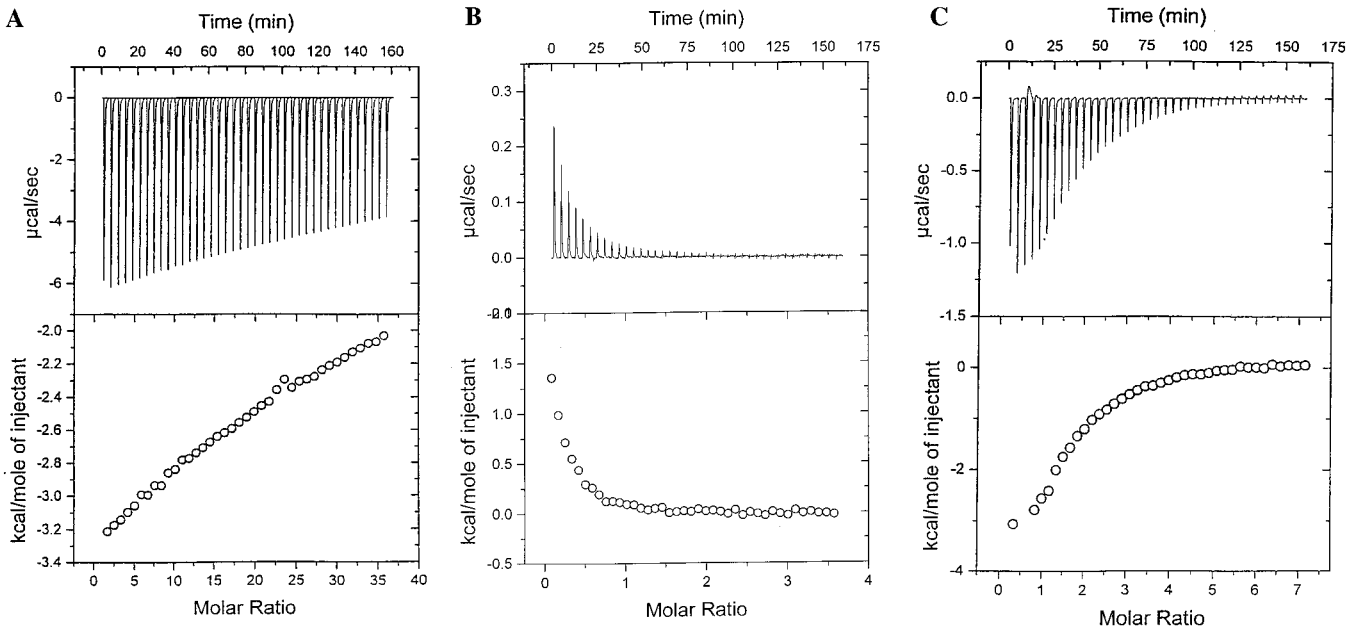

Figure 3. Isothermal titration calorimetry of the interaction of Mg(II) and Eu(III) ions with the GAAA RNA and DNA hairpin loops (see Figure 1A). All experiments were performed in 10 mM MES, 100 mM NaCl, at 25 °C, pH 5.5, in a total volume of 1.4 mL. Raw data are shown in the upper slate and the integrated data is shown in the bottom slate for all figures. (A) Upper panel: 45 μM RNA tetraloop fragment with 40 × 6 μL injections of Mg(II) (50 mM stock solution). Lower panel: the binding isotherm created by integrating under each of the peaks in the top panel and plotted against the molar ratio of metal added to RNA present in the cell; Origin software then fits the integrated data to the appropriate single site binding algorithm to give best values and probable errors from the stoichiometry ($n = 0.95$) binding constant ($K_d = 8$ mM), heat ($\Delta H = 4.2$ kcal mole^{-1}), and entropy ($\Delta S = -13$ e.u) of binding. (B) Titration of Eu(III) with the RNA tetraloop fragment. Upper panel: raw data for 40 × 6 μL injections of 0.5 mM Eu(III) ions into a 50 μM GAAA tetraloop solution. Lower panel: the integrated heats of reaction, $\Delta H = 6.6$ kcal mol^{-1}, $\Delta S = 26$ eu, and $K_d = 6.7$ μM. (C) Titration of the interaction of Eu(III) with the DNA analogue of the GAAA tetraloop. Upper panel: raw data for 40 × 6 μL injections of 0.50 mM Eu(III) ions into 50 μM DNA. Lower panel: integrated heats of reaction, $\Delta H = -7.5$ kcal mol^{-1}, $\Delta S = -4.4$ eu, and $K_d = 38$ μM.

discernible increase in the luminescence intensity was observed upon addition of an excess of Eu(III). Acquisition at an earlier gate (~0.18−0.20 ms) was therefore used for analyzing the Eu(III)−DNA complex. At this gate there was a very modest (less than 10%) increase in luminescence intensity as DNA was titrated into the Eu(III), which was insufficient for accurate determination of values for stoichiometry or affinity.

The decay of Eu(III) luminescence in the presence of the DNA loop analogue was monoexponential, with a constant of 8.97 ms^{-1}, corresponding to fully hydrated Eu(III) ion. Altogether, these data suggest either very weak affinity of Eu(III) for the DNA loop fragment and/or completely water-mediated coordination.

Thermodynamics of Metal Ion Binding. Measurement of ΔH, the enthalpy change upon complex formation, provides important information on the energetic processes as a result of formation or breaking of bonds and removal of waters of hydration. Direct measurement of enthalpic changes upon metal binding was conducted by isothermal titration calorimetry (ITC). Measurements are based on heat exchange and not spectroscopic properties of the ion, thereby permitting direct comparison of the energetics of binding of different metal ions. Also, by maintaining constant temperature, there is the added advantage of avoiding metal-induced RNA cleavage encountered in determination of melting temperature by standard methods.

Representative plots of calorimetric titration of the RNA and the DNA tetraloop fragments with Mg(II) and Eu(III) are shown in Figure 3A−C. The upper panels display the raw data for the metal−RNA titration and the bottom panels represent the integrated heats of reaction for each titration. The binding isotherm, combined with the equation $\Delta G = \Delta H - T\Delta S$, provides a measure of the entropic changes associated with binding. The magnitude of heat production ("negative spikes") or absorption ("positive spikes") in each plot identifies a reaction as exothermic or endothermic, respectively.

Monitoring of the complexation of the RNA tetraloop fragment with Mg(II) indicates that saturation was not reached (Figure 3A), even at Mg(II) concentrations as high as 100 mM (data not shown). An approximate K_d determined from the partial titration was 8 mM, in reasonable agreement with the value obtained from competition with Eu(III) luminescence (13 ± 6 mM). Calorimetrically determined values of ΔH and ΔS were both negative, from which we concluded that the binding is enthalpically driven. These data are in accord with a predominantly outer-sphere binding process, in which the solvated Mg(II) ion forms an ordered complex with RNA through the metal ion's hydration shell.

Titration of Mn(II) into the GAAA RNA fragment (data not shown) resulted in a curve fitted by $\Delta H = -6.9$ kcal mol^{-1} and a K_d of approximately 3 mM, which is essentially the same value as that determined by competition with Eu(III) luminescence.

In contrast, the binding of Eu(III) to the RNA stem loop was characterized by an endothermic, rectangular isotherm with a sharp transition occurring at the stoichiometric equivalence point (Figure 3B). The interaction was characterized by a positive ΔH, +6.5 kcal mol^{-1}, consistent with the removal of waters of hydration upon inner-sphere coordination of Eu(III). The observed dissociation constant, $K_d = 6.7$ μM at 25 °C, from this experiment compares very favorably with the $K_d = 12$ μM previously derived from luminescence studies.[17] From these values, ΔS of +26 cal K^{-1} mol^{-1} was calculated, indicating

that complexation of Eu(III) with the RNA tetraloop is entropically driven, from which we have concluded that the expelled water molecules give rise to solvent reorganizational entropy, which is the major driving force for the binding process.

Titration of the DNA analogue of tetraloop with Eu(III) was then carried out (Figure 3C). Although there had been only a minimal increase in luminescence of Eu(III) upon addition to the DNA stem loop, limiting the information that could be drawn, evidence of binding derived from ITC experiments was unambiguous. The binding isotherm indicated that the binding reaction was exothermic, with $\Delta H = -7.5 \pm 0.29$ kcal mol^{-1} and a K_d of 38 μM. The resulting negligible ΔS of -4.5 cal K^{-1} mol^{-1} suggests that, in contrast to the Eu(III)−RNA interaction, the Eu(III)−DNA binding is enthalpically driven. Taken together with the results of the luminescence decay experiments indicating 9.4 ± 0.5 bound water molecules in the DNA−Eu(III) complex, data from the energetics studies imply that the DNA tetraloop analogue accommodates the hydrated Eu(III) through outer-sphere interactions.

Discussion

We have used luminescence of Eu(III), its competition by other ions, and measurement of calorimetric changes upon RNA−ion complexation by ITC to obtain information about the binding of several di- and trivalent metal ions to a GNRA tetraloop. The ions whose tetraloop-binding properties we have examined include the following: (1) Mg(II), the divalent ion most likely to form complexes with RNA under intracellular conditions; (2) Mn(II), which is isomorphous with Mg(II) but has different chemical properties that translate into different preference for ligands; and (3) Eu(III), which possesses useful spectroscopic properties that permit its binding to RNA to be monitored. Studying an RNA stem loop of known structure and previous documentation of stoichiometric metal-binding behavior in solution provides specific information that will allow study of larger RNA molecules under relatively physiological conditions.

The affinity of Mg(II), derived by either luminescence competition or ITC, was found to be comparable to that derived by competition with cobalt hexammine in NMR experiments.[10] Although Mg(II) is the divalent ion most often bound to RNA, the weak affinity suggests it is not an important contributor to stability of this tetraloop in situ. Rüdisser and Tinoco had concluded that the relatively weaker binding of cobalt hexammine than Mg(II) indicated some inner-sphere coordination of Mg(II).[10] The negative enthalpic change measured upon binding of Mg(II) in our studies suggests that most of the interaction occurs through outer-sphere coordination, i.e. via the existing hydration sphere of the ion. In its predominant form, Mg(II) ion is surrounded by a tightly bound hexahydrate shell, forming an octahedral complex.[33] However, evidence exists for complexes with 4 or 5 waters in the primary coordination shell.[33] In this case, the remaining 1 or 2 water molecules are located in the secondary shell, linked to the primary shell through hydrogen bonding. The high charge:mass ratio of Mg(II) dictates that the first hydration sphere will be tightly bound,[33] but removal of the secondary shell can occur at a minimal enthalpic cost. Therefore, the finding of Rüdisser and Tinoco that cobalt hexammine, an exclusively outer-sphere binding probe, has an even weaker binding is consistent with at least one direct coordination between Mg(II) and the RNA[10] is not necessarily inconsistent with our results. The negative ΔS obtained from our ITC measurements implies an unfavorable entropic cost of ordering waters, which may explain the weak binding.[34]

The weak binding and negative enthalpic change associated with binding of Mn(II) to the RNA tetraloop suggests that its coordination, like that of Mg(II), is predominantly water-mediated. Although different chemical properties mean different preference of ligands, the similarity in data obtained by the two different physical methods suggests that the two ions have overlapping sites.

The metal ions in the lanthanide(III) series, like Mg(II), are "hard" metals and therefore share a preference for phosphate and carbonyl oxygen atoms over nitrogen as inner-sphere ligands. Also, their ionic radius is similar to that of Mg(II) (0.86 and 1.06 Å for Mg(II) and Eu(III), respectively). The tendency of Eu(III) to degrade RNA at temperature above 25 °C[35,36] has been utilized as a tool in the determination of RNA topology;[37] although lanthanide ions have no known biological role, several of them have been found to be useful probes of metal binding sites in biomolecules by NMR, X-ray crystallographic, and luminescence studies. However, unlike the alkaline earth metals, all lanthanides except those with complete f shells are luminescent and paramagnetic.[19] The observation that Eu(III) binds the RNA tetraloop examined in these studies with at least 2 orders of magnitude greater affinity than Mg(II) is commensurate with the incremental binding energy that might be expected for the additional positive charge and additional coordinations (typically 9 vs 6).[38]

Our data from ITC studies (this paper) and from luminescence lifetime measurements[17] demonstrate that nearly the entire hydration shell of Eu(III) is removed upon complexation with the GAAA tetraloop. As a result of its relatively lesser charge: mass ratio than that of Mg(II), Eu(III) has a more flexible and dynamic coordination shell described as a tricapped trigonal prism geometry of C_{3v} symmetry.[39] Accordingly, the cost of losing the inner-sphere water molecules is thus not as pronounced for Eu(III) as it is for Mg(II). Ultrasound relaxation data for the rate of water exchange (k_{ex}) for Eu(III) has been reported to be 6.6×10^{-8} s^{-1}.[40] Eu(III) is known to lose some or all of its waters of hydration more readily than does Mg(II) upon binding of backbone phosphate, base carbonyl, or ribose 2′OH groups.

The DNA analogue of the GAAA tetraloop hairpin has been shown to be less structured,[3,41] lacking the base stacking and the extensive hydrogen boding network found in the RNA.[2] There was no blue shift in the excitation spectrum of Eu(III) bound to the DNA analogue, as was seen for the Eu(III)−RNA complex, and the luminescence lifetime data suggested that the ion was fully hydrated. The energetics of binding, measured by ITC, indicated that the interaction between the DNA analogue

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and Eu(III) is characterized by negative changes in both enthalpy and entropy, also consistent with binding through outer-sphere coordination. These data are in marked contrast with the interaction between Eu(III) and the compact RNA loop fragment, which resulted in highly positive enthalpic and entropic changes.[42] Thus, despite having the same base sequence, differences in positioning of potential RNA groups results in very different metal binding characteristics, further substantiating the supposition of a preformed cage rather than structure induced by availability of base ligands alone. Clearly, the difference in the structure results in different binding behavior and further supports the highly specific binding characteristics of Eu(III) to the RNA tetraloop.

Despite the small size of the RNA tetraloop, we observed heterogeneity in the binding, as evidenced by the finding that the excitation spectrum was well fit by the sum of two Lorentzian−Gaussian curves but not by one unique curve. From this observation, we concluded that there are likely to be two distinct binding microenvironments. The diverse potential coordinating groups apparently allow for the binding of metal ions of differing chemistry and geometry, or for binding of a single ion by more than one set of ligands. Bound ions are not likely to be in rapid exchange between the two sites (on the time scale of this experiment, which has a data acquisition "window" of approximately 20 μs; see the Experimental Section). If this were not the case, we would see a narrow, Lorentzian−Gaussian average of the two instead of the broad peak. We also found that low [Eu(III)] preferentially populated the environment corresponding to the less blue-shifted of the peaks, suggesting that Eu(III) binding was favored at the site. Results of competition experiments with Mg(II) or Mn(II) implied that these ions had no obvious preference for either environment, which suggests that the water-mediated interactions have lesser selectivity. The concept of heterogeneous binding of Eu(III) by a small organic molecule has previously been noted for the crystallographically derived structure of the 1:1 complex formed between Eu(III) or Nd(III) and 3-picoline-*N*-oxide.[43] Although the authors did not comment on it, luminescence excitation spectra of Eu(III) shown in their studies[43] also exhibited two peaks, further supporting the notion that Eu(III) bound to heterogeneous coordination sites displays spectral properties corresponding to the individual microenvironments.

Fluorescence-detected temperature jump relaxation experiments by Menger et al.[44] have indicated two distinct relaxation processes with time constants of tens of microseconds. These results support the existence of more than the single conformation detected by NMR. It is possible that this heterogeneity gives rise to the two binding microenvironments observed in this study. Moreover, flexibility in the local topology may help explain how different ions, having distinct chemical and hydration properties, may bind to the same loop.

We are attempting to characterize the putative binding environments on the basis of several criteria. Excitation at the two resolved peaks resulted in nearly identical luminescence decay constants, leading us to conclude that the heterogeneity is not due to a difference in number of inner- vs outer-sphere coordinations. We then attempted to analyze our data according to empirical equations derived by Wang & Choppin[16] and Frey & Horrocks.[45] These equations have been used to correlate red shifts in the excitation peak of Eu(III) complexes in aqueous solution with the number of negatively charged ionized carboxylate ligands or with the total number of ligands.[45] In neither of these studies, however, were conditions contributing to a blue shift in the excitation wavelength reported. Substitution of our observed shift to the blue into either of their equations resulted in a negative value for the coordination number, suggesting that the approach of these authors is unsuitable for analyzing the Eu(III)−tetraloop RNA coordination. Also, Frey and Horrocks[45] noted that the empirical correlations derived from their equations did not apply to coordination by phosphate groups, which are likely to occur in an RNA−Eu(III) interaction. We are therefore investigating several other possibilities, including altered electronic charge density as a result of metal binding[46] and shifts to higher energies upon sequestering the ion in a more hydrophobic environment.[47]

Solution NMR spectra[39] and circular dichroism spectra,[17] however, do not support any large global change in the structure of the GNRA loop upon binding of the metal ion. However, changes in fluorescence of 2-aminopurine substituted for each of the first two adenine bases in a GAAA tetraloop upon binding of Mg(II) or Ca(II) were consistent with small changes in the stacking of these bases upon binding of metal ion.[44] It is likely that the high level of direct coordination upon binding to Eu(III) similarly induces local changes.

Inspection of the solution structure of the GNRA tetraloop solved by Heus and Pardi[2] reveals that only on the 5′ side of the loop is there a sufficient array of coordination partners available to encapsulate the ion to the extent supported by our data. We speculate that some combination of direct or water-mediated coordinations to phosphate oxygen atoms of G5, A6, or A7 (see Figure 1 for notation); 2′ hydroxyl oxygen atoms of the riboses of C4, G5, or A7; carbonyl oxygen of G5; and/or ring nitrogen atoms (N7/N3 and N1 of adenine) could explain the observed binding behavior without requiring a major alteration in structure. Conversely, the regular base stacking on the helical 3′ side of the loop only allows for coordination to one or two phosphate oxygen atoms, i.e. too few ligands to explain the dehydration observed by luminescence or the energy changes observed upon binding.

The 5′ side of a pentaloop having a GNRA-type fold is also the binding site for the arginine-rich motif of a bacteriophage N protein.[48] Recognition of numerous sites on the major groove edge by polar side chains of the protein (as well as several nonpolar contacts) contributes to the binding specificity, among them the guanosine base (G5 in our loop) and the ribose of the following adenosine (A6). It is not yet clear whether the interaction with Eu(III) has features in common with the binding of the N protein.

Our preliminary model therefore differs from the putative Mg(II) binding site on the 3′ side of the loop identified by

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DeRose and colleagues from changes in the ^{31}P spectrum noted upon addition of the metal ion.[11] Because of differences in the properties of the complex (most specifically, the binding of Eu(III) involves predominantly inner-sphere coordination, whereas Mg(II) is almost fully hydrated), it is always possible that the two metals do not bind in the same site. However, direct competition of Eu(III) luminescence by Mg(II) argues that the two ions bind in the same region of the loop. These authors also observed perturbation in the ^{31}P resonances associated with the 5′ side of the loop, but attributed these changes not to the metal binding site but to evidence for a global change in loop structure associated with the binding. It is anticipated that NMR experiments of this stem loop and its metal ion complexes, currently in progress,[49] will resolve this apparent discrepancy.

In summary, we have explored the binding of Eu(III), Mg(II), and Mn(II) to an RNA tetraloop of the compact and structured GNRA family. The formation of the Eu(III) complex is driven by solvent reorganizational entropy associated with release of most of the inner hydration sphere upon direct binding to RNA ligands. In contrast, Mg(II) or Mn(II) bind through predominantly water-mediated coordination, driven primarily by negative enthalpic changes. Examination of the solution structure of the GAAA tetraloop suggests that the 5′ side of the loop contains a sufficient number of available ligands to encapsulate the Eu(III) ion with the observed specificity without major structural change, and suggests that the tetraloop−RNA complex is an excellent model for detailed study of RNA−metal interaction.

Acknowledgment. This work was partially supported by the Florida State University Council on Research and Creativity. The authors thank Professors Michael Kasha, Gregory Choppin, and Peter Gannett for helpful discussions, the FSU Chemistry Department Laser Laboratory, and the Biochemical Analysis Synthesis & Sequencing Laboratory.

JA012268B

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