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Precise Mapping of RNA Tertiary Structure via Nanometer Distance Measurements with Double Electron−Electron Resonance Spectroscopy

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Many functional RNA molecules fold into specific tertiary structures and may undergo dynamic structural changes that are critical to their function. Adding to static structural information available from X-ray crystallography, advances in biophysical methods using nuclear magnetic resonance (NMR), fluorescence resonance energy transfer (FRET), and electron paramagnetic resonance (EPR) spectroscopies have facilitated determination and prediction of RNA structure and folding.[1] While it is a powerful tool for solution structures and dynamics, macromolecular NMR is challenging for large RNA molecules due to significant spectral overlap and fast signal decay.[2] FRET lacks such biomolecule size limitations and has been used to measure inter-label distances up to 8 nm and to monitor dynamics of biomolecules in single-molecule experiments.[3] As an alternative to FRET that offers very high precision, double electron−electron resonance (DEER) spectroscopy is becoming a popular method for measuring distances between 1.5 and 8 nm as reported by the weak dipolar interaction between radicals.[4] Recently, this method has been applied to functionally important, spin-labeled proteins and naturally occurring radicals.[5,6] DEER measurements have also been reported for nucleic acids, principally for relatively rigid RNA hairpin and RNA or DNA duplexes.[7]

In response to environmental factors such as ionic strength, or to the presence of proteins or small molecules, RNA can undergo complex folding transitions that are mediated by tertiary interactions. In this study, we use DEER distance measurements with site-directed spin labeling (SDSL) to study one such folding event in a metal-induced ribozyme structural change. This work provides an important demonstration of DEER spectroscopy applied to global folding transitions in complex RNA molecules.

The hammerhead ribozyme (HHRz) is a small, self-cleaving RNA motif.[8] The HHRz has three flanking helices around a conserved active-site core and folds into a catalytic Y-shaped conformation in the presence of metal ions.[9] Recent studies have shown that intramolecular loop−loop interactions in native, "extended" HHRz's have a long-range influence on the core, promoting catalytically active conformations and supporting 50- to 500-fold faster cleavage rates than those reported for truncated HHRz's.[9−14] The crystal structure of the *Schistosoma mansoni* HHRz obtained in high ionic strength (Figure 1) shows close interactions between loops I and II.[11a] In solution, global folding of this HHRz has been shown to occur at low Mg^{2+} concentrations,[12,13] but accompanying kinetic measurements find the fastest cleavage rates at higher Mg^{2+} ion concentrations.[12,14] One potential reason for increasing HHRz activity would be further Mg^{2+}-dependent rearrangements in the ribozyme core.

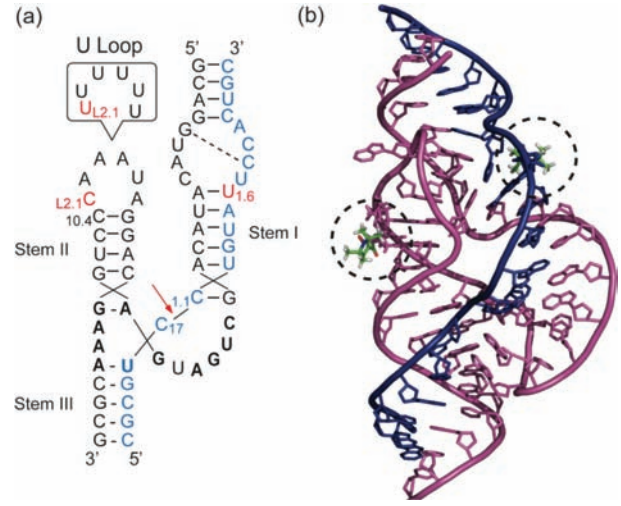

Figure 1. Hammerhead ribozyme (HHRz) structure. (a) Secondary structure of the extended HHRz from *S. mansoni*. The enzyme sequence is written in black, substrate in blue, spin-labeling sites in red, and the conserved nucleotides in bold letters. Ribozyme cleavage site is marked as a red arrow, and the base-pairing in loop I is indicated with a dashed line. The U-loop substitutions are indicated. (b) Model of spin-labeled extended HHRz created from the crystal structure (PDB ID 2GOZ).[11a] Substrate is shown in blue, and the enzyme is in magenta. The spin labels are shown in green with dashed circles.

Here, we investigate Mg^{2+}-ion-induced folding of the extended HHRz from *S. mansoni* by measuring distances between nitroxide spin labels incorporated into stems I and II, and we compare these with ribozyme activity in order to investigate how interstem interactions and tertiary folding are linked to ribozyme catalysis. Two 4-isocyanato TEMPO spin labels[15] were introduced to 2′-amino-substituted $U_{1.6}$ and $C_{L2.1}$ positions in loops I and II of the extended HHRz, respectively (Figure 1a), and ribozyme folding was monitored on the basis of changes in spin−spin distance populations with addition of Mg^{2+} ions. An advantage of studying RNA folding transitions in the HHRz by DEER is that ribozyme activity provides a sensitive assay for functional perturbations by the attached labels. Previously we showed that nitroxide labeling at the 2′-position of U1.6 has little effect on HHRz cleavage rates.[12] Here the activities of ribozymes spin-labeled at both $U_{1.6}$ and $C_{L2.1}$ are found to be nearly identical to those of unlabeled ribozymes (Figure S2, Table S1, Supporting Information (SI)), indicating that labeling at these two positions does not perturb folding to the active structure. As a control RNA, a loop II "U-loop"-substituted ribozyme[12,14] was used, in which the loss of the loop−loop

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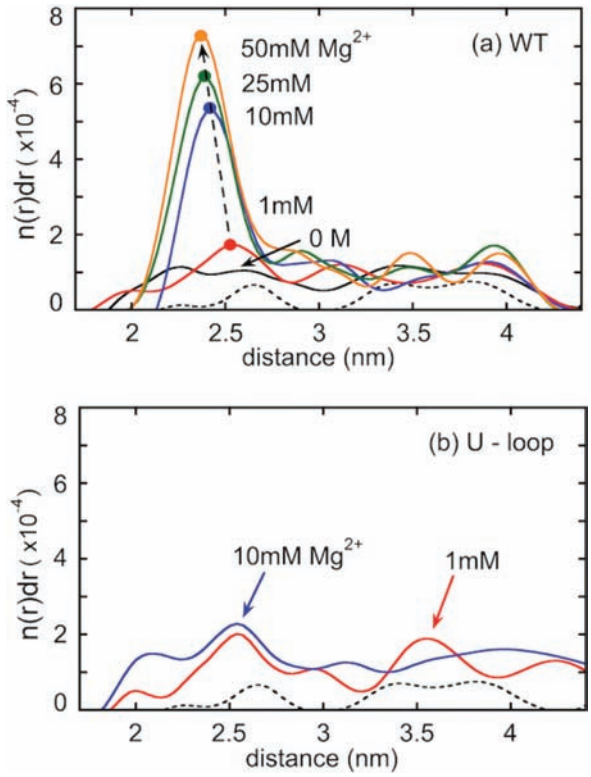

Figure 2. Overlaid distance distribution functions for spin-labeled extended HHRz at various Mg^{2+} concentrations and in 0.1 M NaCl, pH 7.0. Distribution of inter-spin distances for (a) wild-type and (b) U-loop-substituted ribozyme at Mg^{2+} concentrations of 0 (black), 1 (red), 10 (blue), 25 (green), and 50 mM (gold). The black dashed line is for the isolated spin-labeled substrate in 1 mM Mg^{2+} and 0.1 M NaCl.

tertiary interaction strongly decreases activity. For distance measurements, the substrates were protected from cleavage with 2′-OMe modification at C_{17}. Four-pulse DEER traces of frozen-solution samples were obtained, and the data were fit using Tikhanov regularization.[7e]

Figure 2 and Figure S4 (SI) show distance distribution functions for the spin-labeled wild-type (*[$U_{1.6}$,$C_{L2.1}$]) and U-loop-substituted (*[$U_{1.6}$,$U_{L2.1}$]) HHRz's in increasing Mg^{2+} concentrations. Samples containing just a single spin label at $U_{1.6}$ have only background inter-molecular dipolar couplings,[7e] which are then removed in the fitting to reveal only a weak distance distribution (Figure 2, dashed line). This intramolecular distribution fluctuates around zero as a result of noise and imperfections in the theoretical background correction (SI).

In contrast, the double spin-labeled wild-type HHRz shows a strong, metal-dependent increase in the distance distribution at 2−3 nm (Figure 2a). In the absence of Mg^{2+} ions (Figure 2a, black solid line), the distribution between spin labels in the wild-type ribozyme is broad, uniform, and greater than zero, with fluctuations comparable to those of the single-labeled control (dashed line), suggesting that, at low ionic strength, the two stems of the HHRz are randomly oriented. Random stem orientation at low ionic strength has also been predicted from FRET studies.[13b] However, in the labeled wild-type HHRz with 1 mM Mg^{2+}, a small peak appears above the fluctuations and becomes quite prominent at ∼2.4 nm upon addition of up to 50 mM Mg^{2+}. These data show the Mg^{2+}-dependent emergence of a HHRz population with short and defined inter-nitroxide distances.

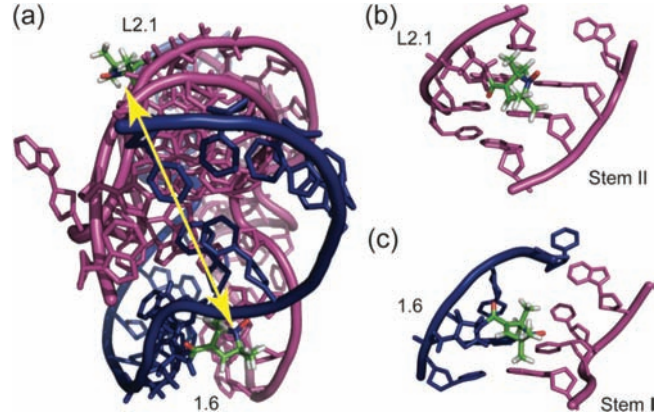

Figure 3. Models of nitroxide label positions based on molecular modeling with HHRz crystal structure (PDB ID 2GOZ, Supporting Information). HHRz enzyme strand is in magenta and substrate strand in blue. Spin-labeling sites 1.6 and L2.1 are shown. (a) View from the top of stems I and II, showing the relative positions of the two labels (green). Close-up views of the label positions in stems II (b) and I (c) depict minor-groove localization that gives excellent agreement between inter-spin distances predicted from modeling and DEER experiments.

A simple model for the nitroxide spin-labeled extended HHRz was constructed from a crystal structure (PDB ID 2GOZ, Figure 3 and Supporting Information) that is assumed to reflect a globally folded form of the RNA. This model predicts an inter-spin distance of ∼2.5 nm between $U_{1.6}$ and $C_{L2.1}$, only for rotamers in which the spin-label motion is restricted by each minor groove. Localization of the 2′-urea-linked spin label at $U_{1.6}$ was also observed in our previous dynamics experiments, which showed immobilization of a single label attached to $U_{1.6}$ upon docking of stems I and II with increasing Mg^{2+} concentrations.[12] Lineshape analysis of those single-label spectra were used to predict Mg^{2+} dependence of RNA folding but could not provide the long-range structural information that is available from these DEER measurements. The agreement between the DEER- and molecular modeling-derived distances of 2.5 and 2.4 Å, respectively, is excellent.

As expected, DEER spectra of the U-loop-substituted HHRz show that the broad distance distributions between labels at $U_{1.6}$ and $U_{L2.1}$ do not change much with up to 10 mM [Mg^{2+}] (Figure 2b). This is consistent with the very low activity observed for the U-loop HHRz and an increase in ionic requirement for folding upon loss of stabilizing tertiary interactions as has previously been measured by FRET.[13b] The U-loop-substituted HHRz data provide an important control showing that the inter-spin population increase at ∼2.5 nm observed in the native HHRz is indeed monitoring increased amounts of the active HHRz structure.

If it is assumed that the increase in short-distance population with higher [Mg^{2+}] reflects an increase in docked HHRz conformation, then the half-maximum [Mg^{2+}] for complete docking between stems I and II can be roughly estimated to be $K_{1/2} \approx 8$ mM (0.1 M Na^+, Figure S3, SI). Global folding of the *S. mansoni* extended HHRz in 0.1 M NaCl has been previously measured by spin-label dynamics and FRET to occur with $K_{1/2} \approx 1$ mM Mg^{2+}.[12,13b] The slightly larger Mg^{2+} requirement for folding exhibited in these DEER measurements could be due to differences in experimental conditions, including the use of cryoprotectant (ethylene glycol) and any shift in equilibrium constant as the DEER sample cooled to the freezing point.

The DEER measurements described here provide precise inter-label distances and can be considered complementary to

measurements obtained by FRET for RNA structure studies. Solution-based FRET measurements can measure structural fluctuations,[3] whereas DEER requires low temperatures and may reflect a distribution of populations. As has been noted,[16] the smaller size of nitroxide labels in comparison with many fluorophores has potential for less perturbation and may allow label placement closer to functionally sensitive regions. Distance determination by this method is robust over a broad range without changing label type, and is free of the problems associated with the relative orientation of transition dipoles in FRET because the localized magnetic transition dipoles are oriented by the magnetic field, not by the molecular structure. The spectral position of a line in EPR is affected by molecular orientation, however, and so it is possible to use non-selective pulses as here, minimizing effects of label orientation, or conversely to exploit selective pulse schemes to gain additional information with highly oriented radicals.[6c]

In summary, the Mg^{2+}-dependent folding of the *S. mansoni* extended HHRz ribozyme has been monitored via observation of nanometer-scale distances between spin labels at $U_{1.6}$ and $C_{L2.1}$ in the two loops I and II using SDSL and DEER spectroscopy. A distinct change due to an increase in the population of ribozymes with a short inter-label distance is observed with increasing [Mg^{2+}]. The measured distance is remarkably consistent with models generated from static crystal structures when it is assumed that the spin labels preferentially localize near the RNA minor grooves. The DEER technique described here can be applied to predict folding of other functional RNA molecules, including within RNA−protein complexes.

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Supporting Information Available: Materials, methods, kinetic data, raw DEER data, and additional references. This material is available free of charge via the Internet at http://pubs.acs.org.

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