SUPPORTING INFORMATION

"Separate Metal Requirements for Loop Interactions and Catalysis in the Extended Hammerhead Ribozyme"

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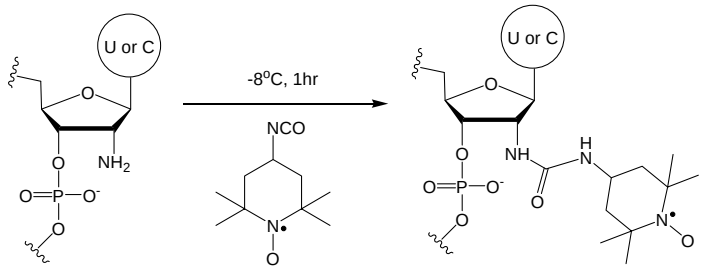
Materials and Methods

RNA Preparation

All RNA strands were purchased from Dharmacon Research Inc. (Lafayette, CO), and deprotected according to the vender's instructions. RNAs were purified by 20% denaturing PAGE, eluted to ×1 TBE from the gel, centrifugally concentrated (Centricon YM-3, Millopore), and ethanol precipitated. The RNAs for the kinetics experiments were dissolved in 5mM HEPES, 100mM NaCl, pH 7.0 buffer. Possible EDTA contamination in the RNA pellet was removed by buffer exchange via centrifugal concentration. The dried RNA pellets for the spin labeling were dissolved in a coupling buffer (70mM boric acid, pH 8.6) and stored at -30°C for the future experiments.

RNA Spin Labeling

The isocyanated nitroxide spin label was synthesized from 4-amino-TEMPO (Aldrich) as described by Edward and coworkers[1]. For the site-directed spin labeling only one nucleotide in the substrate strand was 2'-amino modified as a spin labeling target. 800mM crude spin label dissolved in 8μL of DMF was added to the mixture of 20μL of ~2mM 2'-NH_2 containing substrate and 12μL of formamide for the spin labeling reaction (S.Scheme 1). The reaction mixture was incubated at -8°C for an hour before ×5 chloroform washing. The product was ethanol precipitated overnight and purified with the reverse phase HPLC, and if required, additional 20% denaturing PAGE purification was performed.



S.Scheme 1

EPR Measurements

The mixture of wild type enzyme and spin labeled substrate in 5mM HEPES, 100mM NaCl, pH 7.0 was annealed at 90°C for 90 seconds, fast cooled in ice for 30 minutes. To ensure that all spin-labeled substrate was bound to enzyme, the enzyme:substrate ratio was 2:1. Non-denaturing gel assays and EPR spectral saturation experiments of spin labeled substrates with wild type enzyme strands demonstrate that >80% of the spin-labeled strands are hybridized with enzyme. 80% (w/v) sucrose solution was added into the mixture for final concentration of 30%(w/v) sucrose in order to minimize the global tumbling motion of

the enzyme/substrate complexes after at least 30 minutes equilibrium with the desired amount of $MgCl_2$. 15μL of 150μM wild type enzyme / 75μM spin labeled substrate complex in 5mM HEPES, 100mM NaCl, 30% sucrose, pH 7.0 with desired amount of Mg^{2+} was transferred into 1.5mm (I.D.) capillary tubes. EPR measurements were carried out in a Bruker EMX X-band spectrometer. EPR spectra were collected at room temperature for dynamics measurements with 5 scans over a sweep width of 100G with a microwave power of 5mW, modulation amplitude of 0.8G, time constant of 328 ms, and sweep time of 168 s.

Kinetics Measurements

The nitroxide spin labeled substrates were 5'-end ^{32}P radio-labeled with T4 polynucleotide kinase (USB) for measuring the cleavage rate constant. The radio-labeled substrates were purified with 20% denaturing PAGE. In order to determine the single-turnover rate constant, both hand mixing (all Mg^{2+} concentrations) and rapid quench mixing (≥10mM Mg^{2+}) methods were used. The mixture of 6μM wild type enzyme, 3μM spin labeled substrate and trace amount of radio-labeled spin labeled substrates was annealed in 5mM HEPES, 100mM NaCl, pH 7.0 buffer at 90°C for 90 seconds, fast cooled in ice for 30 minutes, and placed at 20°C water bath. The cleavage reaction was initiated by transferring 2μL of RNA solution into equal amount of Mg^{2+} buffer which contains 5mM HEPES, 100mM NaCl, pH 7.0, quenched by adding 50-300mM EDTA, 80% formamide, and 0.6mg/mL bromophenol blue and xylene cyanol buffer at the given time points, and immediately placed on dry ice. The reaction products were purified by 20% denaturing PAGE, and analyzed by autoradiography using BAS-5000 (FUJI) and Multi Gauge v2.3 (FUJI). The observed rate constants (k_{obs}) fit well with a monophasic equation for 0.05mM Mg^{2+}, and with a biphasic equation for higher Mg^{2+} as observed in recent work from our lab. All calculations and plots were done with Kaledagraph 3.6 (Synergy Software).

$$F = F_0 + F_{max}(1 - e^{-kt}) \text{ (monophasic)}$$

$$F = F_1(1 - e^{-k_1 t}) + F_2(1 - e^{-k_2 t}) \text{ (biphasic)}$$

F: fraction cleaved at time t, F_0: initial fraction cleaved with no Mg^{2+}, F_{max}: maximum fraction cleaved, t: time(minutes), k: observed rate constant for cleavage, F_i: fraction cleaved for population i, k_i: observed rate constant for population i.

For measuring fast kinetics of the natural hammerhead ribozyme, rapid quench flow methods were used (RQF-3 Rapid Quench Flow, Kin-Tek). The annealed solution with 5'-end ^{32}P labeled and site-specific spin labeled substrate/wild type enzyme complexes, and the same amount of Mg^{2+} buffer were loaded in sample loops A and B. They were rapidly mixed in the reaction loop for 0.1-30sec and quenched with the 50-300mM EDTA, 80% formamide buffer. The reaction products were quantified as described above. The time points at 5-15 sec were averaged with data obtained using hand mixing in order to obtain kinetic plots. Detailed calculated parameters for the kinetics experiments are shown in table S3.

The half-maximum concentration of the Mg^{2+} for the cleavage of the hammerhead was calculated using a Hill-type equation.

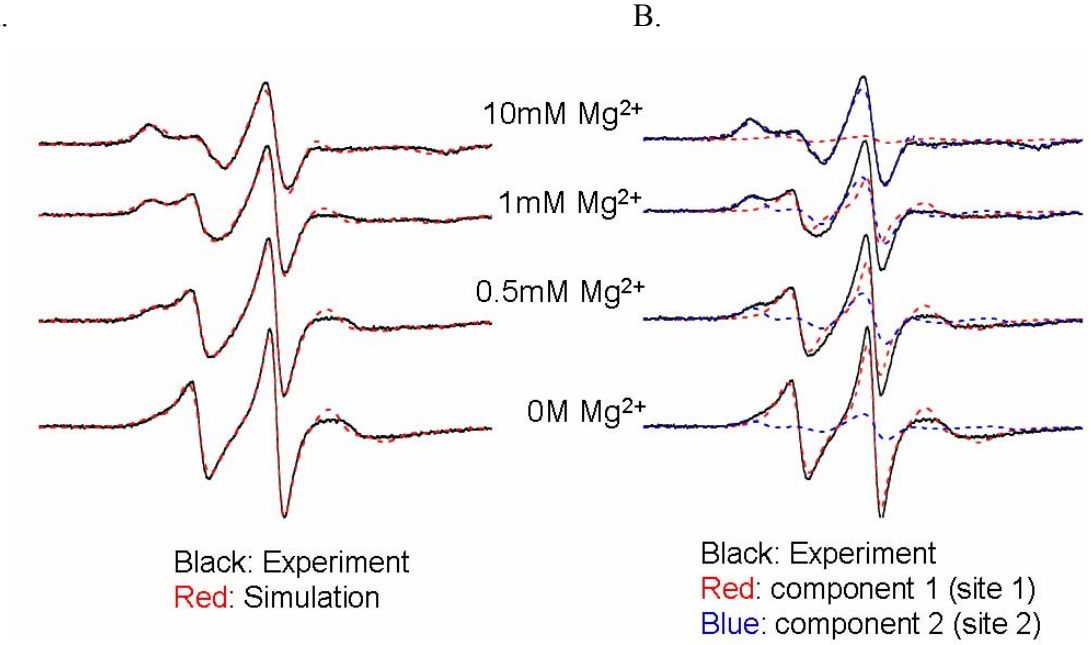
$$k_{obs} = k_{max} \frac{[Mg^{2+}]^n}{[Mg^{2+}]^n + K^n_{Mg^{2+}}}$$

Where, k is the observed rate constant for the cleavage of the hammerhead ribozyme, k_{max} is the maximum rate constant, n is the Hill coefficient, and $K_{Mg^{2+}}$ is the half-maximum concentration of Mg^{2+} or $[Mg^{2+}]_{1/2,cat}$ influencing the cleavage reaction.

EPR Spectra Simulation Using MOMD Model

Experimental spectra for the $U_{1.6}$ spin labeled substrate/wild type enzyme complexes (slS($U_{1.6}$)/wtE) have been analyzed with the microscopic ordering macroscopic disordering (MOMD) model[2] implemented in the NLSL program obtained from the Freed laboratory (ftp://ftp.ccmr.cornell.edu/pub/freed). There are two components in EPR spectra corresponding to a less restricted spin label motion (site 1) and a more restricted motion (site 2). The population of the both sites was calculated from spectra obtained at different Mg^{2+} concentrations. Figure S1A shows the simulated fits to the experimental spectra, and Figure S1B shows the separate components of the fit from site 1 and site 2 at different Mg^{2+} concentrations.

Figure S1.



The parameters used for simulation are shown in table S1.

Table S1. Simulation parameters for the slS($U_{1.6}$)/wtE.

	α_d	β_d	γ_d	$\log R_x$	$\log R_y$	$\log R_z$
Site 1	1.7	36.4	90	5.92	7.60	6.23
Site 2	1.7	23.7	90	6.11	7.41	6.23

g_x = 2.0085, g_y=2.0022, g_z=2.0021, A_x = 5.14G, A_y=5.55G, A_z=37.5G
α,β,γ: rotational diffusion tilt angle, R:rotational diffusion tensor, A:hyperfine parameter

The half-maximum concentration of Mg^{2+} that influences docking of the hammerhead was calculated using the following equation.

$$P = P_o + P_{max} \frac{[Mg^{2+}]^n}{[Mg^{2+}]^n + K^n_{Mg^{2+}}}$$

where P is the population, P_0 is the intrinsic population with no Mg^{2+} in RNA solution, P_{max} is the maximum population, n is the Hill-type coefficient, and $K_{Mg^{2+}}$ is the half-maximum concentration of Mg^{2+} or $[Mg^{2+}]_{1/2,dock}$ influencing ribozyme docking.

Ribozyme activity in 30% sucrose

Activities of the spin labeled hammerhead ribozyme (slS($U_{1.6}$)/wtE) in the presence of 30% sucrose were compared with those without sucrose at several [Mg^{2+}] in order to verify that sucrose does not inhibit the ribozyme activity and that EPR measurements were performed in proper conditions. Figure S2 and Table S2 show that there is no significant change in activities for both conditions suggesting that 30% sucrose does not inhibit the ribozyme activities.

Figure S2. Activities of spin labeled hammerhead ribozyme (slS($U_{1.6}$)/wtE) in the absence (A) and in the presence(B) of 30% sucrose.

A. B.

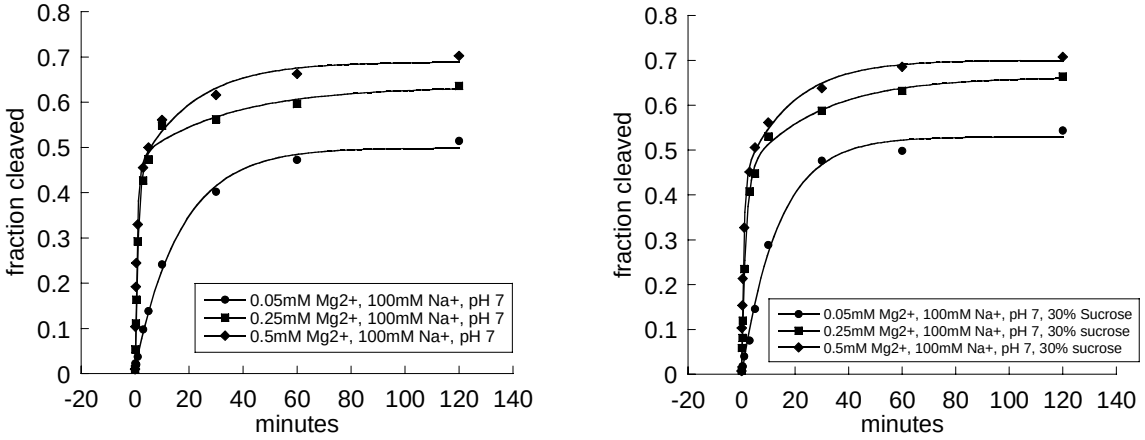


Table S2. Apparent rate constants (k_{obs}) for the spin labeled hammerhead ribozyme (slS($U_{1.6}$)/wtE) cleavage reaction in the presence and absence of 30% sucrose.

[Mg^{2+}] (mM)	No Sucrose, k_{obs} (min^{-1})		30% sucrose, k_{obs}(min^{-1})	
	fast	slow	fast	slow
0.05*	0.06±0.003	n/a	0.07±0.006	n/a
0.25	0.83±0.09	0.03±0.02	0.65±0.06	0.04±0.01
0.5	1.50±0.16	0.05±0.01	1.29±0.09	0.06±0.01

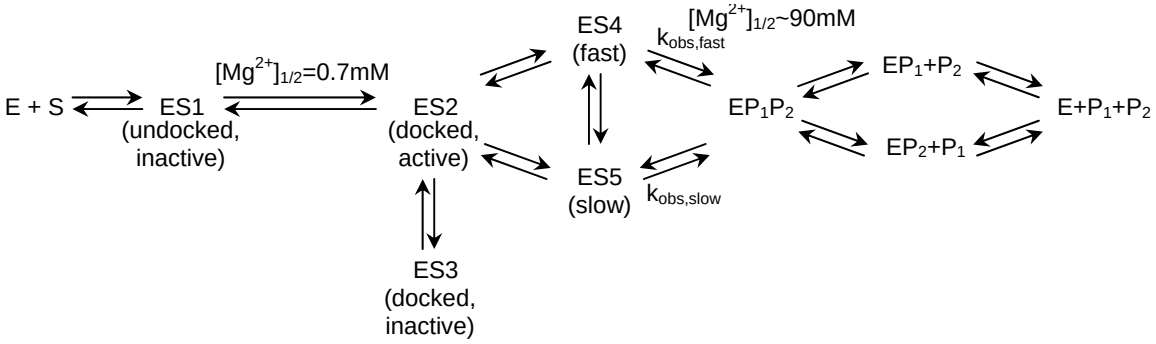
* Data for 0.05mM Mg^{2+} fit well with mono-phasic equation.

Table S3. Kinetic rate constants for slS($U_{1.6}$)/wtE with biphasic catalysis in 5mM HEPES, 0.1M NaCl, pH 7.0 buffer.

$[Mg^{2+}]$ (mM)	$k_{obs,fast}$ (min^{-1})	$k_{obs,slow}$ (min^{-1})	Relative population	
			Fast rate	Slow rate
0.05	0.05 ± 0.003	n/a	1	n/a
0.1	0.19 ± 0.04	0.03 ± 0.01	0.66	0.34
0.5	0.99 ± 0.09	0.04 ± 0.01	0.64	0.36
1	1.5 ± 0.2	0.03 ± 0.01	0.67	0.33
5	3.0 ± 0.3	0.04 ± 0.01	0.70	0.30
10	5.3 ± 0.5	0.13 ± 0.03	0.65	0.35
20	11.3 ± 1.5	0.12 ± 0.04	0.65	0.35
30	13.0 ± 2.3	0.09 ± 0.04	0.65	0.35
50	19.5 ± 3.6	0.26 ± 0.11	0.68	0.32
75	25.3 ± 4.5	0.24 ± 0.10	0.67	0.33
100	27.8 ± 6.1	0.24 ± 0.13	0.69	0.31

Model for a biphasic catalysis of the extended hammerhead ribozyme

One possible model for the hammerhead ribozyme catalysis is established according to the data obtained from our EPR measurements and kinetics experiments as follows.



S.Scheme 2

Increasing [Mg^{2+}] drives the ribozyme to docked conformers (ES2), as shown by the SDSL results. The half-maximum concentration of Mg^{2+} or $[Mg^{2+}]_{1/2}$ for the complete docking is calculated to be 0.7 mM (0.1 M NaCl) from the EPR spectral analyses. Stems I and II of the ribozyme are docked after adding Mg^{2+}, but since the hammerhead ribozyme that has been used for this study does not cleave the substrate strand completely after adding Mg^{2+}(e.g. ~75% cleavage at 1mM Mg^{2+}), there are some inactive docked conformers (ES3) throughout the cleavage reaction. We observe biphasic kinetics over the range of Mg^{2+} concentrations, and the ratio of the populations of fast rate to the slow rate is ~ 2 for all [Mg^{2+}] according to the kinetic data shown in table S3. This indicates that there are two types of active docked conformers, ES4 and ES5, which show fast or slow activities respectively, and there is no conversion of fast and slow conformers throughout the [Mg^{2+}] range. However, the docked conformers ES3, ES4 and ES5 are not distinguishable in our current EPR spectra.

References

1. Edwards, T. E.; Okonogi, T. M.; Robinson, B. H.; Sigurdsson, S. T., Site-specific incorporation of nitroxide spin-labels into internal sites of the TAR RNA; structure-dependent dynamics of RNA by EPR spectroscopy. *J. Am. Chem. Soc.* **2001,** 123, (7), 1527-1528.

2. Budil, D. E.; Lee, S.; Saxena, S.; Freed, J. H., Nonlinear-least-squares analysis of slow-motion EPR spectra in one and two dimensions using a modified Levenberg-Marquardt algorithm. *J. Magn. Reson. Ser. A* **1996,** 120, (2), 155-189.