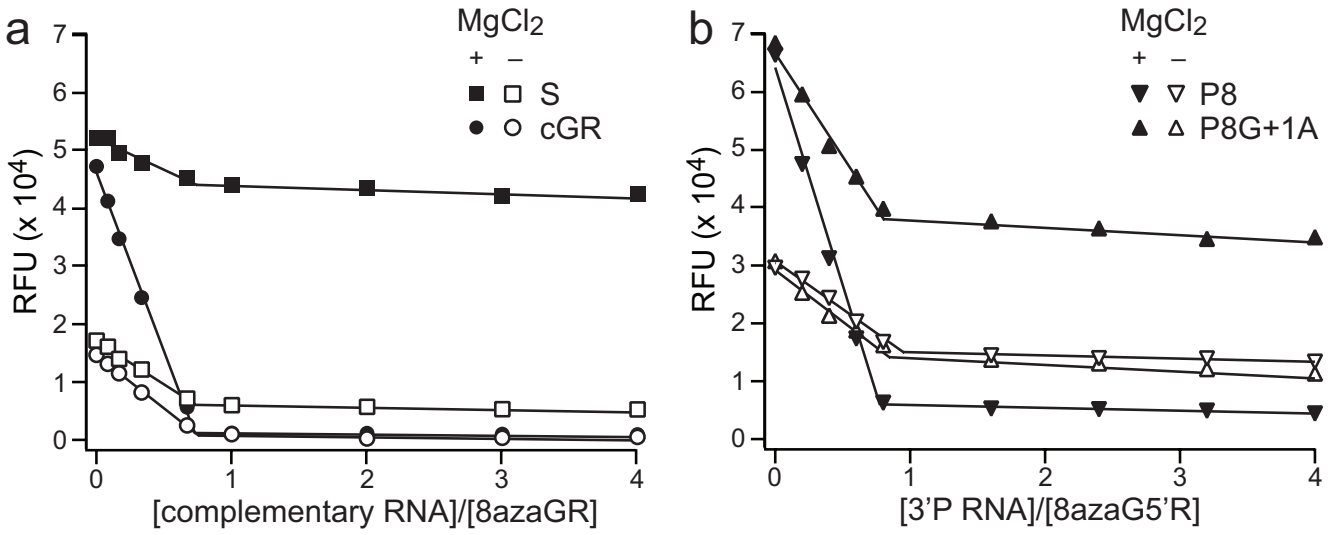
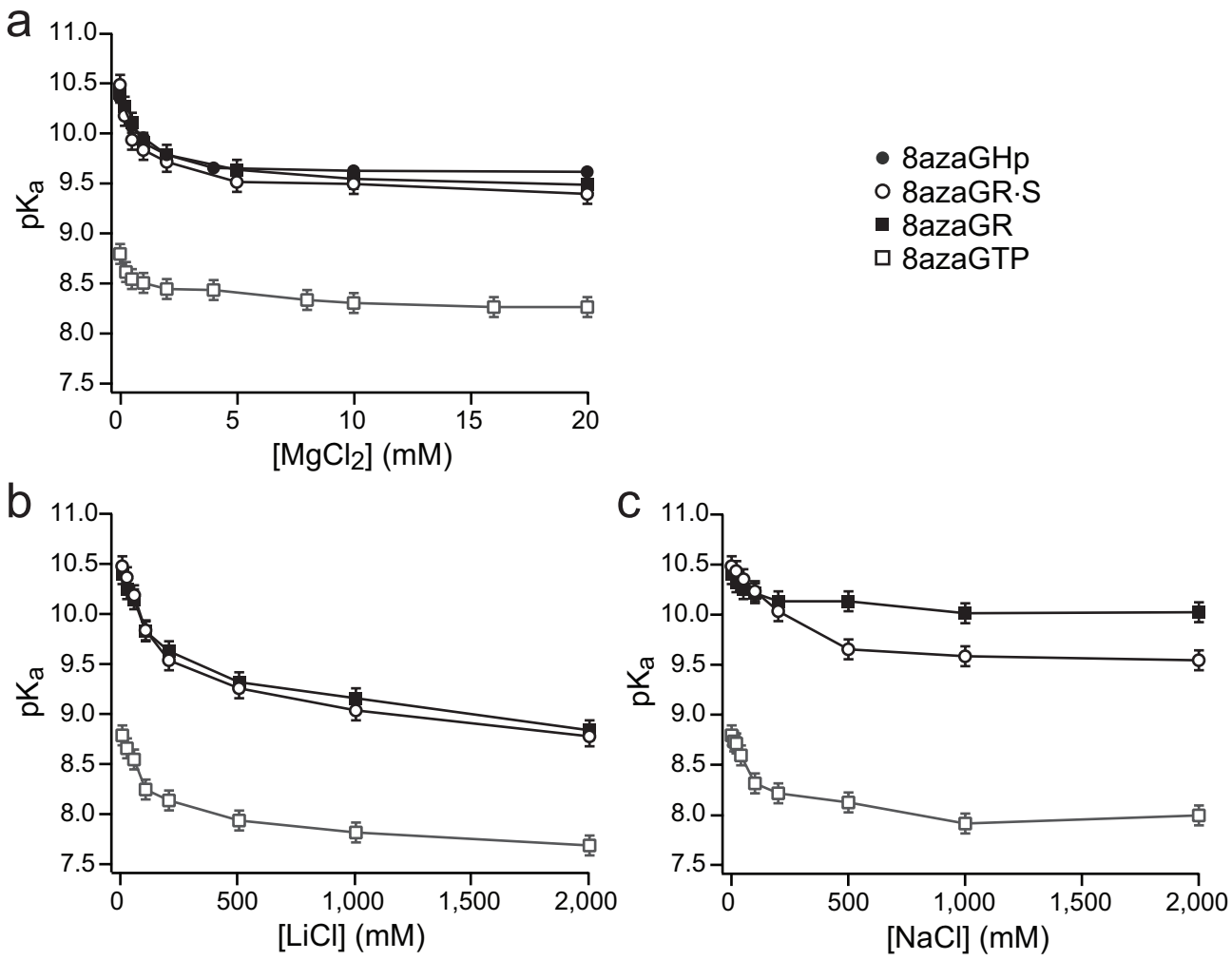
Direct Measurement of the Ionization State of an Essential Guanine in the Hairpin Ribozyme

Lu Liu, Joseph W. Cottrell, Lincoln G. Scott, and Martha J. Fedor*



Supplementary Figure 1. Complex formation with 8azaG RNAs monitored through fluorescence quenching. **(a)** 3 μM 8azaGR RNA was combined with S or cGR RNAs at concentrations ranging from 0 to 12 μM at pH 9.6 by heating at 85˚C for 1 min and slowly cooling to 25˚C in the presence or absence of 10 mM $MgCl_2$. **(b)** 3 μM 8azaG5'R RNA was annealed with P8 or P8G+1A RNAs as described in (**a**).



Supplementary Figure 2. The dependence of microscopic pK_a values for 8-azaguanine deprotonation on salts. Error bars represent standard deviations calculated from two or more measurements.

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SUPPLEMENTARY METHODS

Preparation of RNAs

8azaGR RNA was prepared using T7 RNA polymerase transcription with 8azaGTP, as described previously.[1] Briefly, two synthetic deoxyoligonucleotides (5′ CTA ATA CGA CTC ACT ATA 3′ and 5′ TGG TTG GGT TCT GAT GTT CCT ATA GTG AGT CGT ATT AG 3′) were annealed to form a partially duplex template. Transcription was carried out at 37˚C with 1 μM template in 40 mM Tris-HCl pH 8, 1 mM spermidine, 10 mM dithiothreitol, 0.01% Triton X-100, 80 mg/mL PEG-8000, 0.4 mM 8azaGTP, 12 mM ATP, 9.33 mM CTP, 1.33 mM UTP, 0.4 mM pGpG, and 30 mM $MgCl_2$. After 4 hours, the 8azaGR transcript was fractionated by denaturing gel electrophoresis and converted into a sodium salt by DEAE-650M chromatography (Toyopearl). Transcription of the unmodified GR oligonucleotide was carried out under similar conditions except reactions contained 2 mM GTP instead of 8azaGTP and pGpG.

Since the 8azaGR sequence contains two 5′ terminal guanine residues in addition to 8azaG at position 8, transcription was primed with a pGpG dimer to prevent 8-azaguanine incorporation at the 5' terminus. No transcription products were detected in reactions that contained 8azaGTP without pGpG and a truncated product was observed in reactions that included pGpG but not 8azaGTP, indicating that this strategy was successful in ensuring that the only 8-azaguanine incorporated into 8azaGR was at position 8.

8azaGHPg was prepared through ligation of 8azaGR and a second RNA transcript (ΔAHP48g) that contains the 3' portion of the ribozyme sequence. ΔAHP48g was prepared by T7 RNA polymerase transcription of a linearized plasmid (pTΔAHP48g) as described,[2] and then joined to 8azaGR using T4 DNA ligase and a DNA splint, following published procedures.[3] Briefly, the 5′ triphosphate first was removed from the ΔAHP48g transcript by incubation with Antarctic phosphatase, followed by inactivation of the phosphatase at 65˚C for 10 min. Phosphorylation with T4 polynucleotide kinase and ATP was carried out for 30 min at 37˚C, followed by inactivation of the kinase at 65˚C for 15 min. Next, equimolar amounts of 8azaGR and ΔALR48g were combined with a DNA splint that forms complementary base pairs with 20 nucleotides of each RNA and annealed by heating to 85˚C for 2 min followed by cooling to 60˚C for 2 min and to 37˚C for 5 min. Ligation was carried out at 37˚C for 1 hour in a reaction with each oligonucleotide at a concentration of 2.5 μM, 1 mM ATP, and 20 units/μL of T4 DNA ligase. 8azaGHP48g was then fractionated by denaturing polyacrylamide gel electrophoresis and purified as sodium salts by DEAE-650M chromatography (Toyopearl).

In order to obtain an 8azaG 5'R4g RNA with the appropriate 2',3' cyclic phosphate terminus, a ΔA5′R4 RNA fragment first was prepared by co-transcription of a linearized plasmid template (pTΔAHP43g) that encodes the 3' terminal portion of a ribozyme and the partially duplex template that encodes the GR transcript that provides the 5' terminal portion needed to assemble a complete functional ribozyme. During transcription, GR assembles with ΔAHP43g to form a complete ribozyme

with only three base pairs in H1 to ensure that rapid dissociation of the small 3' product RNA prevents religation and drives self-cleavage to completion. 20 nM linearized plasmid template that encodes ΔAHP43g and 200 nM partially duplex template that encodes GR were combined in a transcription reaction with 2 mM NTPs and 20 mM $MgCl_2$ and incubated at 37˚C for 3.5 hours. ΔA5′R4g was fractionated on denaturing gels and then joined to 8azaGR by DNA-splinted ligation, as described above. ^{32}P-labeled RNAs used in kinetic assays were prepared in the same way except that [γ-^{32}P] ATP was used instead of ATP for phosphorylation of ΔA5′R4g before the splinted ligation step.

SUPPLEMENTARY REFERENCES

1. Da Costa, C.P., Fedor, M.J. & Scott, L.G. 8-Azaguanine reporter of purine ionization states in structured RNAs. *J. Am. Chem. Soc.* **129**, 3426-3432 (2007).

2. Kuzmin, Y.I., Da Costa, C.P. & Fedor, M.J. Role of an active site guanine in hairpin ribozyme catalysis probed by exogenous nucleobase rescue. *J. Mol. Biol.* **340**, 233-251 (2004).

3. Moore, M.J. & Query, C.C. Joining of RNAs by splinted ligation. *Methods Enzymol.* **317**, 109-123 (2000).