Notes

Conformational Dynamics of Self-thiophosphorylating RNA

Bong-Rae Cho* **and Donald H. Burke†**

Department of Applied Chemistry, Division of Applied Science, Cheongju University, Cheongju 360-764, Korea
**E-mail: brcho@cju.ac.kr*
†Department of Molecular Microbiology & Immunology and Department of Biochemistry, University of Missouri, School of Medicine, Columbia, MO 65211-7310, USA
Received November 10, 2006

Key Words : Self-thiophosphorylation, Autokinase, Fluorescence, Cy5

The Kin.46 kinase ribozyme was previously selected from a random-sequence RNA library that was loosely based on the sequence of a known ATP aptamer, based on its ability to catalyze transfer of the thiophosphate from ATPgS to its own 5' hydroxyl end. The reaction requires an oligonucleotide effector that is complementary to the 3' end of the ribozyme and that served as reverse transcription primer during the amplification steps of the original selection for activity (Fig. 1).[1,2] Omitting the oligonucleotide reduces the observed catalytic rate constant (k_{obs}) by 10^3 to 10^6-fold, indicating that the oligo acts as an allosteric effector that is necessary for full catalytic activity. The activator helix is separated from the substrate-binding internal guide sequence by a 5nt "linker" that appears to form long-range base-paring interaction with nucleotides within the catalytic core (S. Rhee, unpublished results).

Binding of small ligands, such as metal ions and ATP, can also potentially influence the folded structure of the RNA. Divalent metal ions are well known for their abilities to stabilize nucleic acid secondary and tertiary structures, and for their potential catalytic roles in ribozyme-catalyzed reactions. For example, we recently found that topologically rearranged versions of Kin.46 exhibit markedly altered Mg^{2+} dependence.[3] Similarly, nucleotide binding by aptamers nucleates formation of the folded structure and induces order in the NTP binding pocket (adaptive binding).[4-8] ATP recognition by Kin.46 is clearly distinct from ATP recognition by the original aptamer; key nucleotides required for ATP recognition are mutated in Kin.46, and the values of K_m^{ATPgS} and K_m^{ATP} for Kin.46 (~3 mM) are $>10^3$ times higher than K_d^{ATP} for the aptamer (~1 μM). In this work, fluorescence emission from cy5-labeled ribozymes was monitored in order to investigate conformational dynamics as a function of ATP concentration and Mg^{2+} ion.

Truncated versions of Kin.46 ribozyme were obtained by internal cleavage or deletions (Fig. 1). Ribozyme130 was derived from the Kin.46 by just cleaving the most right-side loop, ribozyme119 was obtained by deletion of the loop and

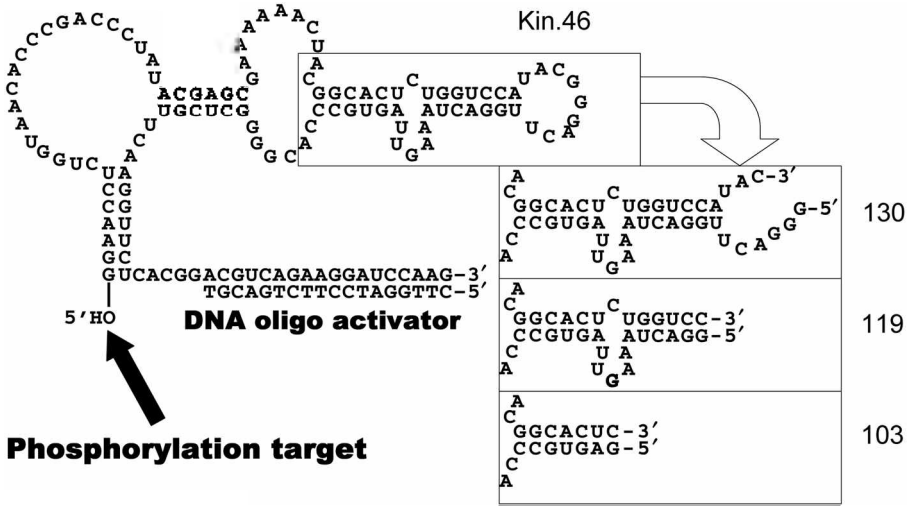

Figure 1. Predicted secondary structure of Kin.46 self-thiophosphorylating ribozyme. This kinase ribozyme catalyzes the transfer of the γ-thiophosphate from ATP-γ-S to its own 5'-end. This ribozyme has DNA oligonucleotides with 18 bases complementary to the 3' primer binding sequence (PBS) used in the amplification steps during the original selection for activity. Truncated versions of kinase ribozyme were derived from Kin.46 by internal cleavage or deletions.

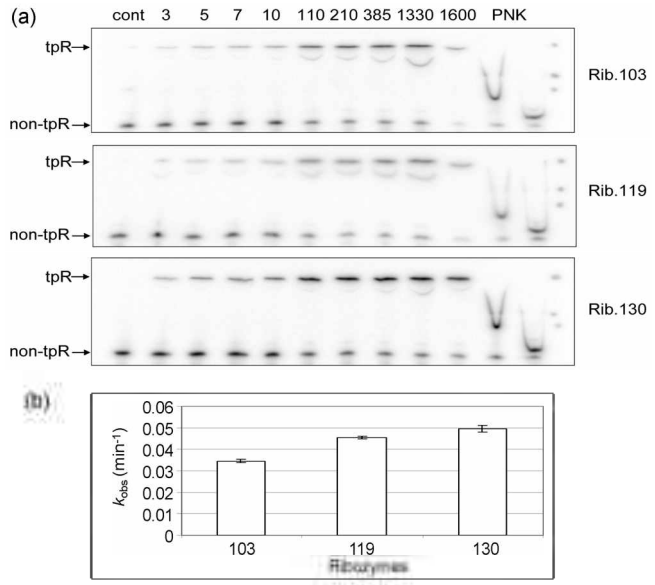

Figure 2. (a) The thiophosphorylation reaction of three ribozymes (103, 119 and 130) was initiated by addition of ATPγS to 10 mM at RT. Aliquots were removed at different times and ribozyme thiophosphorylated (tpR) with ATPγS were separated from nonthiophosphorylated ribozyme (non-tpR) within [(N-acryloylamino)-phenyl] mercuric chloride (APM) polyacrylamide gel. The extent of thiophosphorylation was estimated by exposure to storage phosphor screens and the data were fit to a kinetic equation; the fraction normalized at time t = $(f_\infty - f_0)(1-\exp(-k_{obs}t))$. "Cont" indicates ribozyme not having undergone thiophosphorylation. PNK lane treated with polynucleotide kinase was also applied to normalize each lane. Reaction times (min) are denoted above each lane. (b) Comparison of observed rate constant (k_{obs}(min^{-1})) of truncated ribozymes. Error bars are shown.

ribozyme103 was got by deletion of the most right stem-loop region. Catalytic activities of each ribozyme were compared with organomercurial polyacrylamide gel electrophoresis (oPAGE) by which to monitor auto-thiophosphorylation by ribozymes (Fig. 2). The thiophosphorylated product migrates more slowly based on the strong interaction between mercury and sulfur.[9,10,11] Therefore, truncated versions of Kin.46 were incubated with ATP-γ-S and the products were separated by oPAGE using gels that contained [(N-acryloylamino)phenyl] mercuric chloride (APM). Their activities did not vary so much, suggesting that the most right-side single strand loop does not play an important role in the ribozyme activity. As the large single-strand which has 20 nts, joining the seven nucleotides to the rest of the ribozyme can be severed or omitted to yield a multiple turnover, trans-activating enzyme,[2] ribozyme was assembled from four different strands: a 7nt RNA substrate (7-mer), a 31nt "upper" strand (up), a 63nt "lower" strand (lw) and an 18nt activating oligomer (AO18) (Fig. 3). Fluorescent dye, cy5-labeled AO18 was purchased from IDT (Integrated DNA Technologies).

Four-stranded ribozyme was assembled with cy5-AO18 and analyzed by native gel electrophoresis to monitor conformational heterogeneity. The label migrates to a single, major band, with very small amounts of an additional band

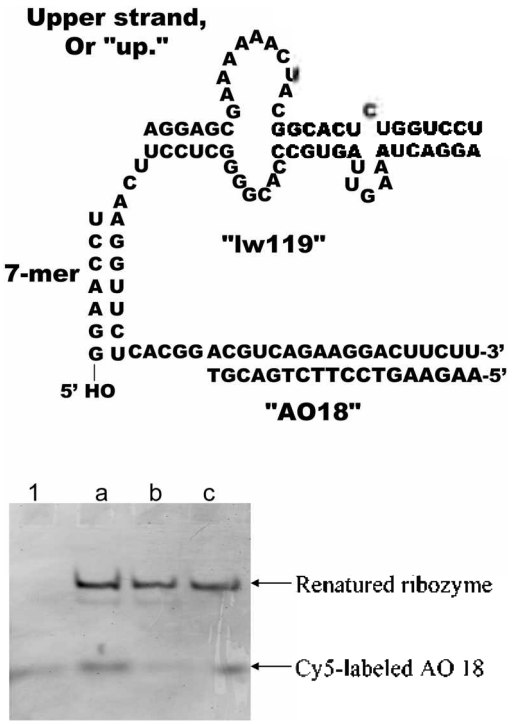

Figure 3. A major conformer of a ribozyme composed of four strands. Four strands (4 uM of 7-mer, 4 uM of up strand (up), 4 or 8 uM of lower strand (lw) and 4 or 8 uM of cy5-labeled activating oligomer (cy5-AO) with 18 nucleotide) were heated at 90 °C for 2 min and allowed to cool to RT (~21 °C). The mixtures were subject to 8% native PAGE after the treatment of microcon with 30 nucleotide cut-off for single stranded RNA/DNA. Lane 1, only cy5-labeled AO18 (4 uM); Lane a, ribozyme composed of cy5-labeled AO18 (4 uM), lw strand (4 uM) and up strand (4 uM); Lane b, ribozyme composed of cy5-labeled AO18 (4 uM), lw strand (4 uM), up strand (4 uM) and 7-mer (8 uM); Lane c, ribozyme composed of cy5-labeled AO18 (4 uM), lw strand (4 uM), up strand (8 uM) and 7-mer (4 uM).

of higher mobility (Fig. 3). These results suggest that the four-stranded version of Kin.46 folds predominantly into a single, active conformation after renaturation.

The binding of ATP to RNA and DNA aptamers induces significant conformational change in the aptamers.[4-8] To determine whether a similar conformational change occurs in this self-phosphorylating ribozyme, Kin.46 was assembled with cy5-AO18 and emission spectra were measured as a function of ATP concentration in the absence of Mg^{2+} (Fig. 4). Fluorescence emission from cy5-AO18 progressively decreases as ATP concentration is increased when the label is in the context of the ribozyme, but not for the AMP-Cy5-AMP dye by itself. These data support a model in which ATP binding induces a conformational change in Kin.46 in such a way as to perturb the environment of the cy5 dye. Mg^{2+} ion was also added in the range of 2.5 mM to 47.5 mM in the presence of 12 mM ATP and the decrease of the emission was observed.

Divalent metal ions are known to stabilize the secondary and tertiary structures of RNA, and mutations that perturb Mg^{2+} binding sites often affect thermal stability.[12-14] Kin.46

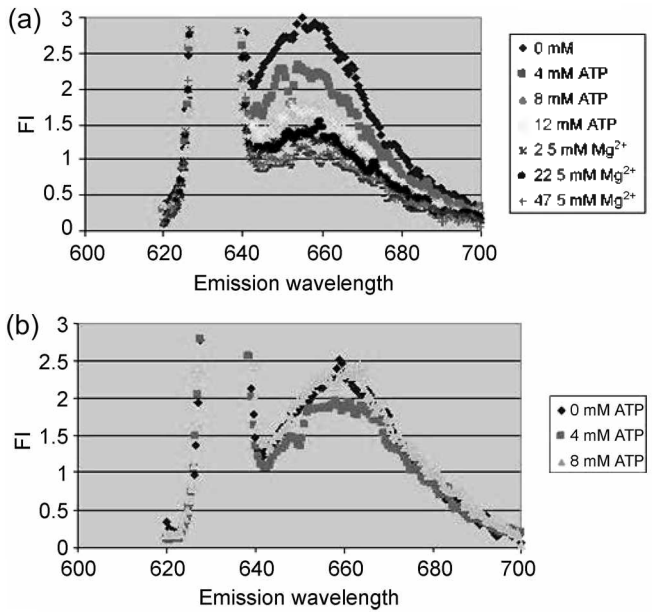

Figure 4. Emission scanning of (a) a ribozyme with cy5-labeled AO18 (12 pmol), lw strand (12 pmol), up strand (14 pmol) and 7-mer (12 pmol), and (b) cy5 dye only with excitation at the absorbance wavelength of cy5 (633 nm). Emission fluorescence was scanned at the excitation wavelength of cy5, 633 nm under the condition of Ex slit (nm) : 5.0, Em slit (nm) : 5.0 and scan speed (nm/min) : 100 with LS-50B Luminescence Spectrometer (Perkin Elmer). The ATP concentration ranges is 0 mM to 12 mM without $MgCl_2$ and $MgCl_2$ is also added in the range of 2.5 mM to 47.5 mM in the presence of 12 mM ATP. ◆ represents 0 mM ATP, □ 4 mM ATP, Δ 8 mM ATP, × 12 mM ATP, * 2.5 mM $MgCl_2$, • 22.5 mM $MgCl_2$ and + 47.5 mM $MgCl_2$.

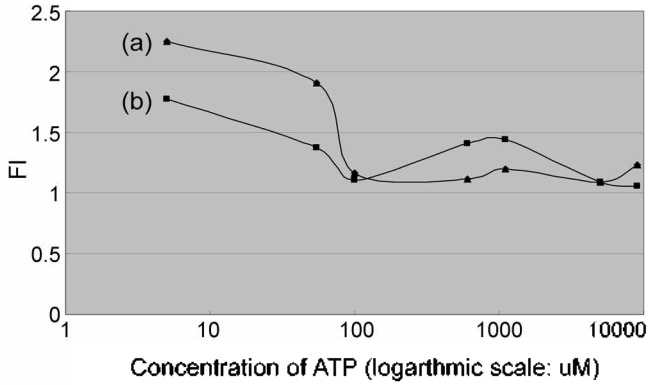

Figure 5. Emission scanning of a ribozyme composed with 4 strands as a function of ATP concentration at the constant Mg^{2+} concentration of (a) 5 mM and (b) 50 mM. Emission fluorescence was scanned at the same condition as described previously. Emission intensities at 659 nm, the maximum emission wavelength of cy5 were compared as a function of logarithmic scale of ATP concentration.

was originally selected in 50 mM Mg^{2+}. To determine the effect of magnesium on the ATP dependence of the conformational change, fluorescence emission was monitored as a function of ATP at both 5 mM and 50 mM Mg^{2+} (Fig. 5). Emission intensities were compared at 659 nm (maximum value for cy5 emission). At 5 mM Mg^{2+}, there is a clear decrease in fluorescence emission as ATP concentrations were increased, again supporting the possibility of an ATP-driven conformational change. In contrast, no such ATP-dependent fluorescence decrease is observed when a similar set of spectra were measured at 50 mM Mg^{2+}. A plausible explanation for this last observation is that the higher concentration of Mg^{2+} drives the conformational change to near completion even before any ATP is added. Alternatively, folding may be synergistic with Mg^{2+}, such that transition occurs at much lower ATP concentration.

In conclusion, conformational change of a ribozyme derived from Kin.46 self-thiophosphorylating ribozyme by internal deletions was detected by measuring emission fluorescence of cy5 in context of ribozyme and appears to be induced by ATP in the presence of Mg^{2+} in a two-state, cooperative transition.

Experimental Section

Kinetic assay of ribozyme. Internally radiolabelled up strand using [α-^{32}P] UTP, 7-mer and lw strand of ribozyme, and activating DNA oligomer (AO18) were heated in KCl/Pipes buffer (200 mM KCl in 150 mM Pipes-KOH, pH 7.0) at 90 °C for 2 min and allowed to cool to RT (~21 °C). These were adjusted to a final concentration of 50 mM $MgCl_2$ and preincubated for 15 min at RT. The thiophosphorylation reaction was initiated by addition of ATPγS to 10 mM at RT. Aliquots were removed at different times and the reaction quenched with 94% formamide, 30 mM EDTA (pH 8.0) containing xylene cyanol and bromophenol blue. Thiophosphorylated ribozymes were separated from nonthiophosphorylated by electrophoresis in APM polyacylamide gel in 90 mM Tris-borate (pH 8.3) and 2.5 mM EDTA containing 7 M urea. The extent of thiophosphorylation was estimated by exposure to storage phosphor screens and imaging (Molecular Dynamics). The data were fit to a kinetic equation; the fraction normalized at time t = $(f_\infty - f_0)(1-\exp(-k_{obs}t))$, where k_{obs} is the observed rate constant.

Confirmation of a major conformer of ribozyme. Four strands for a ribozyme (4 uM of 7-mer, 4 uM of up strand, 4 or 8 uM of lower strand and 4 or 8 uM of cy5-labeled activating oligomer (cy5-AO) with 18 nucleotide) were renatured in the same KCl/Pipes buffer as above. The mixtures were subject to 8% native PAGE after the treatment of microcon with 30 nucleotide cut-off for single stranded RNA/DNA.

Emission scanning of ribozyme with 4 strands. Four strands for a ribozyme (12 pmol of 7-mer, 14 pmol of up strand, 12 pmol of lower strand and 12 pmol of cy5-attached activating oligomer (cy5-AO) with 18 nucleotide) were renatured as above. Emission fluorescence of this ribozyme was scanned at the excitation wavelength of cy5, 633 nm under the condition of Ex slit (nm) : 5.0, Em slit (nm) : 5.0 and scan speed (nm/min) : 100 with LS-50B Luminescence Spectrometer (Perkin Elmer) after the addition of a various concentrations of $MgCl_2$ (5 mM and 50 mM) or ATP (0 mM to 9 mM).

References

1. Lorsch, J. R.; Szostak, J. W. *Nature* **1994**, *371*, 31.
2. Lorsch, J. R.; Szostak, J. W. *Biochemistry* **1995**, *34*, 15315.
3. Cho, B.; Burke, D. H. *RNA* **2006**, *12*, 2118.
4. Sassanfar, M.; Szostak, J. W. *Nature* **1993**, *364*(6437), 550.
5. Dieckmann, T.; Suzuki, E.; Nakamura, G. K.; Feigon, J. *RNA* **1996**, *2*(7), 628.
6. Jiang, F.; Kumar, R. A.; Jones, R. A.; Patel, D. J. *Nature* **1996**, *382*(6587), 183.
7. Huizenga, D. E.; Szostak, J. W. *Biochemistry* **1995**, *34*(2), 656.
8. Lin, C. H.; Patel, D. J. *Chem. Biol.* **1997**, *4*(11), 817.
9. Igloi, G. L. *Biochemistry* **1988**, *27*, 3842.
10. Rhee, S. S.; Burke, D. H. *Analytical Biochem.* **2004**, *325*, 137.
11. Saran, D.; Nickens, D. G.; Burke, D. H. *Biochemistry* **2005**, *44*(45), 15007.
12. Guo, F.; Cech, T. *Nat. Struct. Biol.* **2002**, *9*, 855.
13. Fang, X.; Srividya, N.; Golden, B.; Sosnick, T.; Pan, T. *J. Mol. Biol.* **2003**, *330*, 177.
14. Kim, K.; Choi, W.; Gong, S.; Oh, S.; Kim, J.; Kim, D. *Bull. Korean Chem. Soc.* **2006**, *27*(5), 657.