Proximity of Both Ends of Stems P3 and P4 of Self-kinasing RNA by ATP

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The Kin.46 kinase ribozyme was selected for the transfer of the thiophosphate from ATPγS to its own 5' hydroxyl end. The reaction requires an oligonucleotide effector that is complementary to the 3' primer binding sequence (PBS) of the ribozyme and this effector served as reverse transcription primer during the amplification steps of the original selection for activity (Fig. 1).[1,2] It was found that the oligonucleotide acts as an allosteric effector that is necessary for full catalytic activity. The activator helix formed by the PBS and the oligo-effector is connected by a 5nt "linker" region to the substrate-binding internal guide sequence and stabilizes a long-range base-paring interaction between the 5 nucleotides of the linker and the catalytic core which ATP binds to.

Binding of ATP, a substrate of Kin.46, can potentially influence the nucleic acid secondary and tertiary structures in ribozyme-catalyzed reaction. 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 $K_m^{ATP\gamma S}$ and K_m^{ATP} for Kin.46 (~3 mM) are > 10^3 times higher than K_d^{ATP} for the aptamer (~1 mM).

Fluorescence Resonance Energy Transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule. The donor is the dye that initially absorbs the energy and the acceptor is the chromophore to which the energy is subsequently transferred. FRET is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity.[9-13] In this work, FRET from two dyes (TMR and cy5) - labeled ribozyme was monitored in order to investigate conformational dynamics, especially the relative approach between end of stem P3 and that of stem P4 of Kin. 46 ribozyme as a function of ATP.

Truncated version, a ribozyme119 of Kin.46 was obtained by deletion of the most right-side loop for labeling of each different dye to two different ends (stem P3 and P4) of ribozyme (Fig. 1). The activity of ribozyme119 monitored with organomercurial polyacrylamide gel electrophoresis (oPAGE) which contained [(N-acryloylamino)phenyl] mercuric chloride (APM) did not vary so much,[14] 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 nucleotides, 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).) TMR (tetramethylrhodamine) was labeled to the 5' end of a lower strand with post-synthetic labeling of thiol-modified RNA. Cy5-labeled AO18 was purchased from IDT (Integrated DNA Technologies).

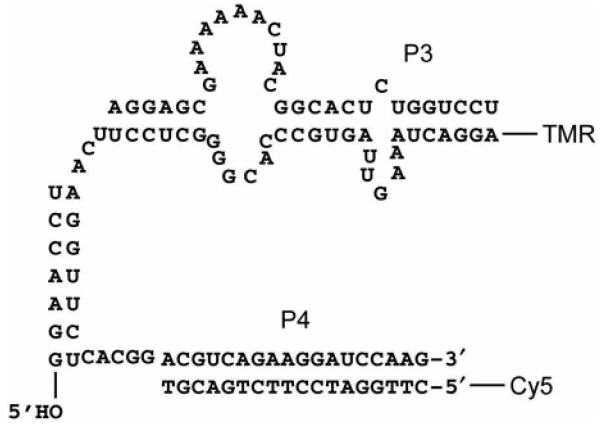

Figure 1. Secondary structure model of ribozyme 119 derived from Kin.46 self-thiophosphorylating ribozyme by internal deletions. This kinase ribozyme catalyzes the transfer of the γ-thiophosphate from ATP-γ-S to its own 5'-end. This ribozyme has DNA oligonucleotides with 18 bases complementary to the 3' primer binding sequence (PBS) used in the amplification steps during the original selection for activity. TMR was labeled at 5'-end of lower strand RNA and Cy5-labeled AO18 was purchased.

The binding of ATP to RNA and DNA aptamers induces significant conformational change in the aptamers.[4-8,15,16] To determine whether a conformational change, especially the relative approach between end of stem P3 and that of stem P4 occurs in this self-phosphorylating ribozyme, four-stranded ribozyme was assembled with TMR-lower strand and cy5-AO18, and emission spectra were scanned after addition of ATP (Fig. 2). Quenching was observed on addition of ATP and then Fluorescence emission from donor dye, TMR, instantly decreases but the emission from acceptor

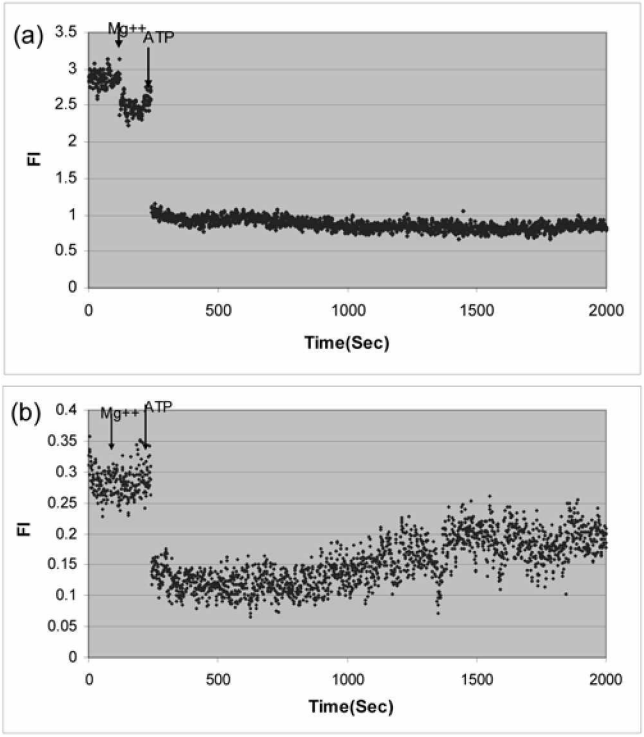

Figure 2. Fluorescence emission scanning of ribozyme119 with cy5-labeled AO18 (12 pmol), TMR-labeled lw strand (12 pmol), up strand (14 pmol) and 7-mer (12 pmol). Excitation was occurred at the absorbance wavelength of TMR (543 nm). Emission fluorescence was scanned at both the emission wavelength of TMR, 585 nm(a) and the wavelength of cy5, 668 nm(b) 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) soon after the addition of 4 mM ATP.

dye, cy5-AO18 increases. These data support a model in which ATP binding induces a conformational change in Kin.46 in such a way as to bring the both ends of stems P3 and P4 close. This complies with the previous result that topological rearrangement connecting both ends of stems P3 and P4 yields structural stabilization.[3] But fluorescence emission difference from both the donor and acceptor did not change symmetrically. A plausible explanation for this observation is that there are 13 base pairs in helix P3 but 18 base pairs in helix P4. It is recommended that the number of nucleotides between helix P3 and P4 is identical for the best result of FRET experiment.

In conclusion, conformational change of a ribozyme119 derived from Kin.46 self-thiophosphorylating ribozyme by internal deletions was detected by measuring emission fluorescence of both donors, TMR, and acceptor, cy5, for FRET. The result suggested that both ends of stems P3 and P4 of the ribozyme come close by the addition of ATP.

Experimental Section

Preparation of TMR-labeled RNA strand. A lower strand was prepared by run-off *in vitro* transcripton with T7 RNA polymerase from the DNA template to which the T7 primer was annealed and purified by PAGE (polyacrylamide gel electrophoresis) containing 7 M urea. The gel-purified lw strand was dephosphorylated with CIP (calf intestinal alkaline phosphatase) and thiophosphorylated at 5'-end with ATPγS and T4 polynucleotide kinase. Thiol-modified RNA pellet was dissolved in 5.5 μL of H_2O, followed by addition of 37.5 μL of 100 mM HEPES-KOH, pH 7.0. The mixture was then transferred to the fluorophore solution made by dissolving 100 μg of tetramethylrhodamine-5-iodoacetamide dihydroiodide in 7 μL of DMSO (dimethyl sulfoxide) and shaken overnight at RT with wrapped in aluminum foil. TMR-labeled lw strand was then purified by PAGE.

Fluorescence emission detection of ribozyme119 with 4 strands. Four strands for ribozyme119 (12 pmol of 7-mer, 14 pmol of up strand, 12 pmol of TMR-labeled lower strand and 12 pmol of cy5-attached activating oligomer (cy5-AO) with 18 nucleotide) 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). This ribozyme was excited at the excitation wavelength of TMR, 543 nm and then fluorescence emission was detected at both the wavelength of 585 nm for the donor and the wavelength of 668 nm for the acceptor 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) soon after addition of 4 mM ATP.

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