Efficient Substrate Cleavage Catalyzed by Hammerhead Ribozymes Derivatized with Selenium for X-ray Crystallography

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Supporting Materials

General Information

Most solvents and reagents were purchased from Fluka, Sigma, or Aldrich (p. a.) and were used without purification unless otherwise noted. Ampliscribe T7 RNA transcription kit by Episcenter was used for transcription of the hammerhead ribozymes. Pyridine was dried and distilled over KOH. Dioxane was dried using sodium metal and benzophenone, and was subsequently distilled. All solid reagents used were dried under high vacuum for 12 hours prior to use. All chemical reactions were performed under Argon, except acid neutralization and NaCl/EtOH precipitation. RNA transcription and digestion reactions were performed in air. ESI-TOF High Accuracy MS analysis was performed at the Scripps Research Institute, California. The top strand and template DNAs were synthesized by DNA synthesizer, and RNA substrate 33.1 was purchased from Dhamarcon Research, Inc. All gel electrophoresis experiments were run using12.5% polyacrylamide gel at constant power of 5 W for 1.5 hr. Gels were all fixed with 10% solution of acetic acid in methanol, and dried under vacuum at 80 ^{o}C for 2 hr. Quantitation of radioactive gels was performed on BioRad phosphoimager.

Synthesis and Purification of NTPαSe

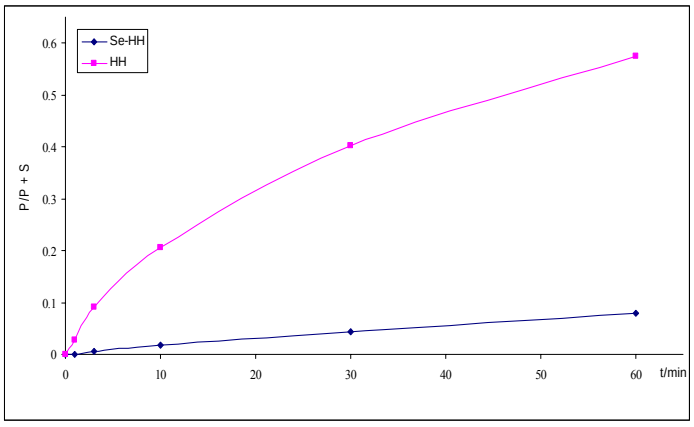
All NTPαSe compounds were synthesized *via* a modification of TTPαSe, ATPαSe, and NTPαS synthesis (reference 17, 18, 21). A general procedure is described here. The starting material, 2',3'-diacetyl nucleotides (0.100 mmol, dry), was placed in a round bottle flask (5 mL). Pyridine (0.200 mL) and dioxane (0.200 mL) were added into this flask to dissolve the starting material. This solution was then injected drop-wise over 5 min into a round bottle flask (20 mL) containing 2-chloro-4H-1,3,2-benzodioxaphorphorin-4-one (20.3 mg, 0.100 mmol, 1 eq.) in dioxane (0.300 mL). The reaction mixture was stirred at room temperature for 1 hour. Afterwards, a solution of pyrophosphate (64.1 mg, 0.14 mmol, 1.4 eq.) in anhydrous DMF (0.300 mL) and tributylamine (0.050 mL) was injected into the reaction mixture, and the reaction mixture was stirred for 20 min. A solution of 3H-1,2-benzothiaselenol-3-one (43.0 mg, 0.2 mmol, 2 eq) in dioxane (0.300 mL) and triethylamine (0.050 mL) was injected into the reaction mixture. After the reaction mixture was stirred for 30 min., ammonium hydroxide (conc., 3.0 mL) was injected into the reaction mixture and reaction mixture was stirred overnight for 8 hr at 45 ^{o}C. After the pH of the reaction mixture was adjusted to pH 7.5-8.0 using 80% acetic acid, the crude mixture was transported to a 50 mL plastic tube and centrifuged at 4000 rpm for 15 min to remove Se metal. The supernatant was collected in two new 50 mL plastic tubes (10 mL each), and NaCl solution (3.0 M, 1.1 mL) and EtOH (thoroughly purged with Ar, 33 mL) were added to each tube. After the solutions were placed in -80 ^{o}C freezer for 10 minutes, they were centrifuged at 4000 rpm for 15 min. The supernatant was discarded, and the pellet was dried on high vacuum. Each solid sample was dissolved in water (1 mL), and combined. NaCl/EtOH precipitation was repeated two more times, decreasing the volume each time. Finally, the crude product was dissolved in water (0.250 mL).

The crude product was purified on RP-HPLC using an Agilient Zorbax C18 column (9.4 mm x 250 mm). The Se-modified triphosphate products were eluted (6 mL/min) using a linear gradient from 100% buffer A [10 mM triethylammonium acetate (TEAA)] to 2.5% buffer B (50% acetonitrile in 10 mM TEAA) in 20 minutes. The diastereomers were analyzed by RP-HPLC and MS. HPLC and HR-MS analyses of UTPαSe diastereomers were shown in Figure 2.

Activity Study of the Se-Hammerhead Ribozymes using Mn^{2+} as the Metal Cation

Ribozymes were transcribed as previously discussed without the addition of any radioactive NTPs, and were desalted by centrifugation using a membrane (3000 Dalton cut-off) three times. The ribozymes were concentrated and adjusted to the same concentration on the basis of the time-course transcription experiment. A cocktail containing the Tris-Cl buffer (pH 7.6, 10 mM $MnCl_2$) and the ^{32}P-labeled RNA substrate was made, and it was split into 5 equal portions. The ribozymes transcribed with ATPαSe I, CTPαSe I, GTPαSe I, UTPαSe I, and NTPs were individually added to each portion to initiate the substrate digestion reaction incubated at 27 ^{o}C. Aliquots (3.5 μL each) were removed from the reaction mixture at the corresponding time points and quenched individually with the loading dye (3.5 μL) containing EDTA (100 mM), followed by placing on dry ice. The digestion reactions were analyzed by PAGE (Figure S1).

A



B

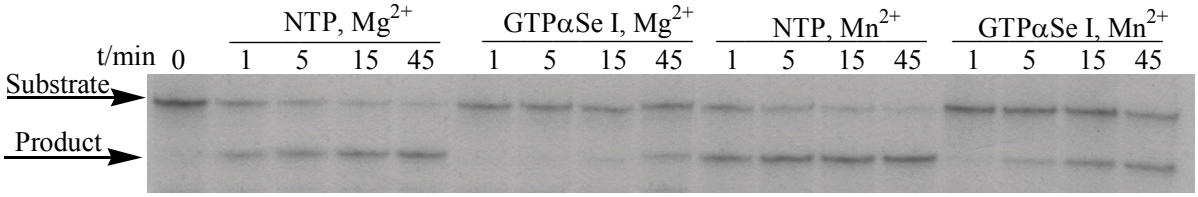


Figure S1. Catalysis of the modified and native hammerhead ribozymes: (A) The time-course Mn^{2+} rescue experiment of the ribozymes transcribed with ATPαSe I and ATP (also see Figure 4C). (B) The time-course Mn^{2+} rescue experiment of the ribozymes transcribed with GTPαSe I and GTP.