

A Small Aptamer with Strong and Specific Recognition of the Triphosphate of ATP

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Experimental Detail

Chemicals and Materials

Adenosine 5'-monophosphate and adenosine 5'-triphosphate (linked via a 9-atom isourea spacer at the C-8 position of adenine) agarose, ATP, ADP, AMP, GTP, CTP, ATP γ -S, α - β methyleneadenosine 5'-triphosphate (α - β methylene ATP), β - γ methyleneadenosine 5'-triphosphate (β - γ methylene ATP), adenosine 5'-(β - γ -imido) triphosphate (β - γ imido ATP), inosine 5'-triphosphate, dATP and cordycepin 5'-triphosphate (3'dATP) are from Sigma-Aldrich. Diaminopurine triphosphate and 7-deazaadenosine triphosphate are from TriLink Biotechnologies. Microcon YM-30 filters are from Amicon. Superscript II reverse transcriptase is from Invitrogen.

The AMP and ATP agarose resin was prepared by rehydrating in water and washing several times. The resin was stored in water and equilibrated with wash buffer (300 mM NaCl, 10.1 mM MgCl₂, 0.1 mM EDTA, and 25 mM Tris pH 7.8) prior to use.

RNA Pool

The starting random RNA pool used to select all aptamers in this study was derived from a random dsDNA pool termed Pool A, which contains 5×10^{15} unique sequences and whose construction has been detailed previously¹. The dsDNA pool has two constant regions flanking a 70 nucleotide random region. The constant regions provide a promoter for T7 RNA polymerase-mediated transcription for each round of selection as well as primer binding sites that allow for reverse transcription and polymerase chain reaction (RT-PCR) amplification of the recovered sequences. The RT-PCR product is the template for RNA transcription for the next round of selection. The constant regions are: *GCTAATACGACTCACTATAGGGAGATCACTTACGGCACC* and *CCAAGGCTCGGGACAGCG*, with the T7 RNA polymerase promoter in italics. In round 1, each transcript had a length of 110 nts (40 nts constant and 70 nts random).

***In Vitro* Selection Scheme**

For round 1, approximately 50 nmol of radiolabeled RNA was transcribed from 0.5 μ M Pool A dsDNA in transcription buffer (2.5 mM spermidine, 5 mM each NTP, 10 nM α -³²P-UTP, 10 mM DTT,

25 mM MgCl₂, 0.01% Triton X-100, and 10 mM Tris, pH 7.4) at 37°C for 4 hrs with 20 U/μl T7 RNA polymerase. For subsequent rounds, 12 nmol of RNA was synthesized under similar conditions. Full length RNA was then purified by denaturing (8M urea) polyacrylamide gel electrophoresis and eluted from the gel with an Elutrap electrophoresis chamber from Schleicher and Schuell Bioscience. The RNA was kept in wash buffer throughout the selection procedure. For ATP and AMP elutions steps, 5 mM ATP or AMP was added to wash buffer, and the MgCl₂ concentration was adjusted to compensate for chelation of free Mg²⁺ by the nucleoside phosphates.

Each selection round (Supporting Figure 2) began with a negative selection against AMP binders, in which the RNA was brought to a concentration of 5 μM in wash buffer and first passed over an AMP-linked agarose column. The bed volume of the AMP resin was 3 mL for round 1 and 1.0 mL for subsequent rounds. The RNA that flowed through this column was then passed over an ATP-linked agarose column. The bed volume of the ATP resin was 0.75 mL for round 1 and 0.25 mL for subsequent rounds. The flowthrough from the ATP column was discarded and the RNA bound to the ATP column was washed with 10 column volumes (CV) of buffer, followed by 6 CV of buffer containing 5 mM AMP and 2 CV wash buffer. Finally, ATP binders were specifically eluted with wash buffer containing 5 mM ATP. The eluted RNA was then amplified by RT-PCR and that material was used as the template for the next round of selection.

Cloning and Sequencing

Individual cloned sequences were obtained by ligation of the RT-PCR material into a bacterial expression plasmid using the TOPO TA Cloning Kit (Invitrogen), per the manufacturer's directions. Individual bacterial clones were grown in 7mL of LB media containing 100 μg/mL ampicillin overnight at 37°C. Plasmid DNA was recovered with the QIAquick miniprep kit (Qiagen). Sequencing of the cloned DNA was performed by the Massachusetts General Hospital DNA Core Facility (<https://dnacore.mgh.harvard.edu/index.shtml>).

ATP and AMP Column Binding Assays

For each clone, approximately 500 μl of 1 μM radiolabeled RNA was incubated in wash buffer with either 200 μl of ATP- or AMP- linked agarose (final bed volume) for 30 min in a Bio-Rad Poly-Prep Chromatography Column. The columns were then washed with 12 CV of wash buffer. The remaining RNA was eluted from the column with 10 CV of wash buffer containing 5 mM ATP. The pooled initial RNA flowthrough and wash fractions, ATP elution fractions, and the AMP and ATP agarose resin were then analyzed with a Beckman LS6500 scintillation counter (Supporting Figure 4).

ATP Column Elution Assays

For each clone, the RNA was incubated with ATP-linked agarose as above. After 30 min, the input was allowed to flow through the column and collected. The column was then washed with 12 CV of wash buffer. The RNA bound to the column was then eluted with 6 CV of 5mM AMP buffer followed by 2 CV wash buffer alone. Next, the RNA was eluted with 6 CV of 5mM ADP buffer and 2 CV of wash buffer alone. The RNA was then eluted with 6 CV of 5mM ATP buffer and 2 CV of wash buffer alone. These fractions and the ATP agarose resin were then analyzed with a Beckman LS6500 scintillation counter (Supporting Figure 5).

Solution binding assays

For all competitive binding experiments, the 1-1min aptamer was used at 15 μM, the concentration determined to give 60-75% binding to radiolabeled ATP in solution in the absence of competitor. For each data point, 200 μl of 1-1min aptamer in wash buffer was incubated with a trace amount of γ-³²P-ATP and an increasing concentration of unlabeled competitor, as indicated. The reaction was allowed to reach equilibrium for 4 hrs. Each sample was then transferred to a Microcon

YM-30 filter and centrifuged for 5 sec to prime the filter. The filters were then transferred to new tubes and centrifuged for 16 sec. Precisely 15 μ l were taken from above and below the filter, representing the total amount of radiolabeled ATP (i.e., free labeled ATP and labeled ATP bound to the aptamer) and free radiolabeled ATP only, respectively. The percent of total radiolabeled ATP bound to the aptamer was then calculated and plotted against the unlabeled competitor concentration.

K_d determination

The solution binding data using ATP as the unlabeled competitor were fit to the following binding equation:

$$Y = (B/(PT - (((PT+X+B) - ((PT + X + B)^2 - 4PTX)^{.5})/2)) + 1)^{-1}$$

Where Y = the fraction of labeled ATP bound to aptamer, B = the dissociation constant, P = the aptamer concentration, X = the unlabeled competitor concentration and T = the percentage of the aptamer concentration that is correctly folded.

For competition by other competitors, the following equation was used:

$$Y = (K/(PT - (((PT+X+B) - ((PT + X + B)^2 - 4PTX)^{.5})/2)) + 1)^{-1}$$

Where K = the determined dissociation constant for ATP

Additional Results

Classification of Cloned Sequences

At round 8 of the selection, ~23% of the input RNA specifically eluted from the ATP column with 5 mM ATP (Supporting Figure 3). Cloning of this material yielded 35 total sequences (Supporting Table 1). Of these sequences, 12 contained a stem loop with a conserved loop motif (5'-GAAGGAGUC-3') and a conserved linkage between 2 stems (5'-UCAG-3'). These clones were termed Class 1, and yielded seven distinct sequences: 1-1 through 1-7. The 23 remaining clones (Class 2) were represented by seven unique sequences (2-1 through 2-7), which had no similarity to Class 1 or to each other. Interestingly, clone 2-1 was the most abundant, appearing in 13 of the 35 sequences. However, its binding activity was not optimal, and it was not studied further (see below).

Screening of Clones for activity

Each clone was analyzed for specific binding to ATP by incubation with either an ATP- or AMP-linked agarose column (Supporting Figure 4). The data indicate that none of the Class 1 clones bound to the AMP column, while all clones bound to the ATP column ~40-50%. Class 2 clones also did not bind to the AMP column. However, clones 2-5, 2-6 and 2-7 did not bind the ATP column, and were therefore no longer considered. The remaining clones bound to varying degrees. In particular, 50% of the clone 2-1 RNA was in the ATP elution fraction, but an additional 20% remained on the column and could not be specifically eluted with free ATP. These data suggest that 2-1 may preferentially bind to C-8 linked ATP.

We also tested the ability of the clones to be specifically eluted from the ATP column by free ATP by incubating them with ATP agarose, and eluting them sequentially with AMP, ADP and finally ATP (Supporting Figure 5). Clone 2-1 showed very high discrimination for ATP elution versus ADP or AMP. However, in solution it failed to bind ATP to an appreciable degree (data not shown), and was therefore excluded from further study. Other Class 2 clones had a similar profile. For most Class 1 clones, more RNA was eluted with ATP than with ADP or AMP, as expected. However some clones (1-3, 1-5 and 1-7) showed little or no discrimination. Clone 1-1 showed the highest discrimination and was used for further study.

References

(1) Urbach, J. M. In *The Department of Chemistry and Chemical Biology*; Harvard University: Cambridge, 1999, p 159.

Supporting Figure Legends

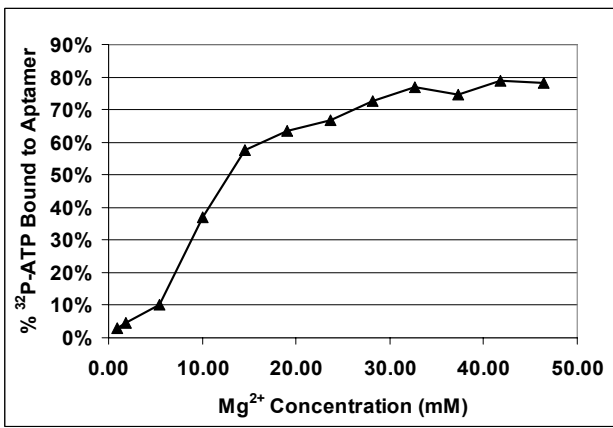
Supporting Figure 1. Magnesium dependence of ATP binding by 1-1min aptamer. The percent of radiolabeled ATP bound to the 1-1min aptamer in the presence of varying Mg^{2+} concentrations and no unlabeled competitor is plotted.

Supporting Figure 2. The aptamer selection scheme used in this work.

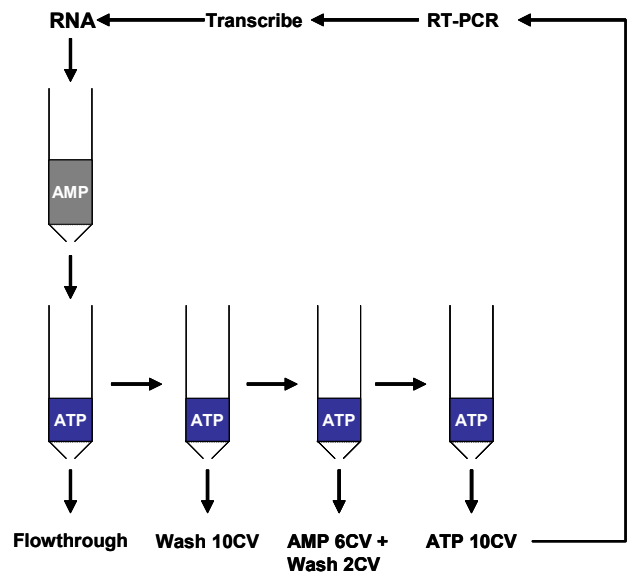
Supporting Figure 3. Percentage of total RNA eluted from the ATP selection column during each round of selection.

Supporting Figure 4. Binding of Clones to AMP and ATP agarose columns. For each clone, the RNA was incubated with an AMP or ATP column for 30 min. The flowthrough and the 12 CV wash with wash buffer were collected together (Wash). RNA was eluted from the column by 10 CV of 5 mM ATP in wash buffer (ATP). The RNA remaining on the column was also quantified (Column).

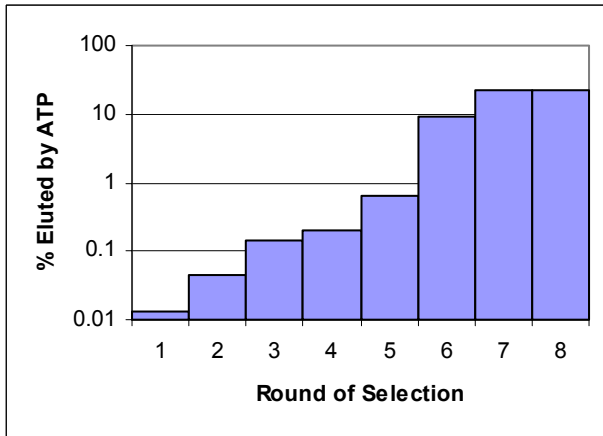
Supporting Figure 5. Elution of Clones from an ATP column. For each clone, the RNA was incubated with an ATP column for 30 min. The flowthrough (Flow) and the 12 CV wash with wash buffer (Wash) were collected separately. RNA was eluted from the column sequentially with 6 CV of AMP (AMP), ADP (ADP) and ATP (ATP), with 2 CV of wash buffer rinses between each specific elution. The RNA remaining on the column (Column) was also quantified.



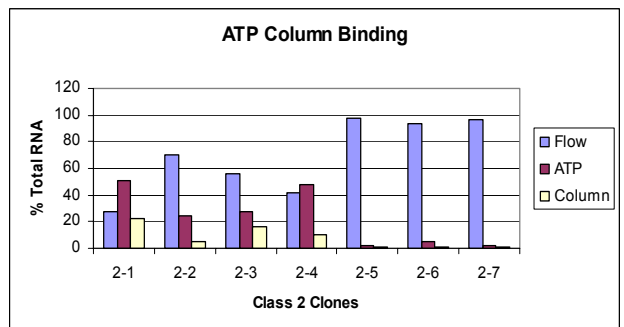
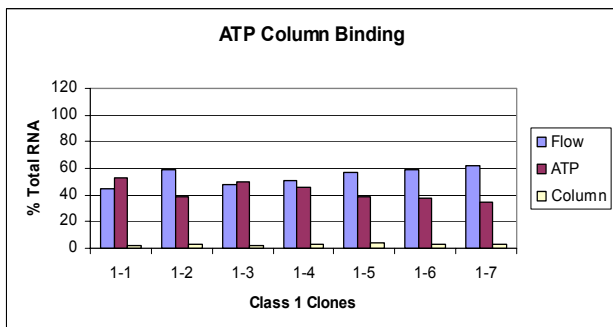
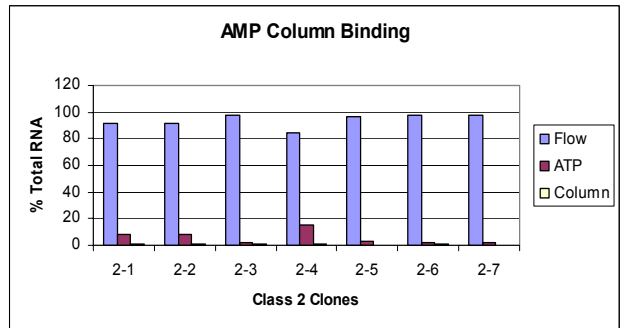
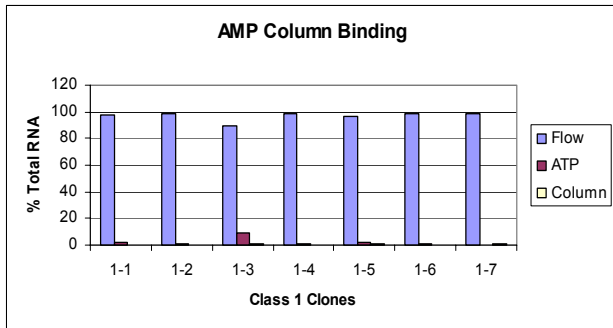
Sazani and Szostak. Supplemental Figure 1



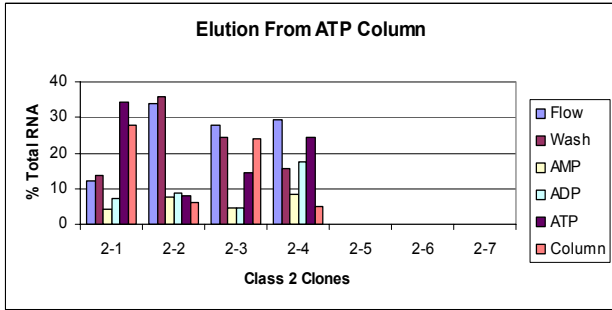
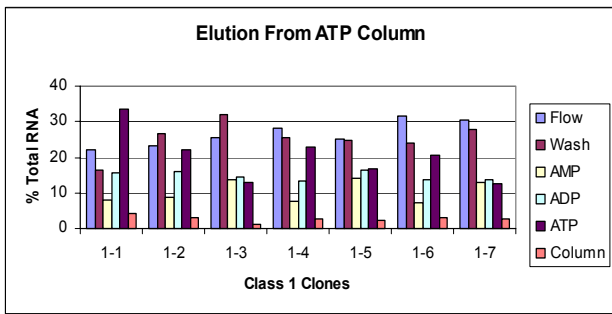
Sazani and Szostak. Supporting Figure 2



Sazani and Szostak. Supporting Figure 3



Sazani and Szostak. Supporting Figure 4



Sazani and Szostak. Supporting Figure 5