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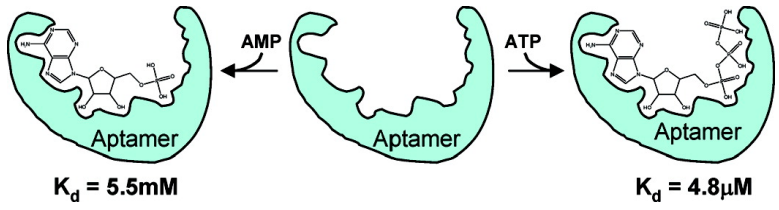

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A Small Aptamer with Strong and Specific Recognition of the Triphosphate of ATP

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In vitro selection has yielded aptamers that bind to a diverse array of targets,[1,2] often with high affinity and specificity.[3,4] However, questions remain concerning the ability of RNA to recognize certain targets, especially anionic moieties. It is clear from studies of NTP-binding aptamers that the nucleobase is the preferred recognition target, with fewer and less significant interactions observed with the sugar and especially the triphosphate.[5−8] Indeed, NMR structural analysis of one ATP aptamer shows that the triphosphate is directed out of the binding pocket and into the surrounding solution.[9,10] One RNA enzyme[11] (ribozyme) and two messenger RNA riboswitches[12] from *Escherichia coli* are known to discriminate between differently phosphorylated small molecule substrates. For the ribozyme, these interactions contribute only ∼1 to 2 kcal/mol of binding energy. The riboswitches show greater discrimination, but seemingly at the expense of a long sequence and complex structure. Here we address the question of whether small RNA aptamers are capable of strong and specific interactions with a triphosphate moiety by selecting small motifs that bind ATP but not AMP.

The initial RNA library consisted of 5×10^{15} unique sequences, each with 70 randomized positions. The experimental scheme involved a negative selection step against molecules that bind to AMP immobilized on agarose beads, followed by a positive selection step for RNA molecules that could bind to immobilized ATP. Furthermore, RNAs that bind to AMP were also selectively discarded from the ATP column by washing with buffer containing 5 mM AMP before the remaining RNAs were competitively eluted with 5 mM ATP. This ATP-specific material was amplified by RT-PCR and T7 transcription to provide the input RNA for the next round of selection. After four rounds of selection, the RNA population exhibited ATP-specific binding.

Fourteen unique sequences were seen among 35 RNAs cloned from the seventh round of selection. Approximately half of these sequences (class 1) contained two highly conserved sequence motifs; sequence alignment (Figure 1) showed that these motifs were embedded in a common secondary structure. Of these RNAs, clone 1−1 showed particularly efficient (50%) binding to immobilized ATP but not to immobilized AMP (2%); furthermore, this RNA was eluted from the ATP resin with free ATP but not AMP (see Supporting Information). We designed a minimized version of the 1−1 aptamer, termed 1−1min (Figure 2), that contained only those structural and sequence elements common to all of the independent class 1 aptamers. The 57 nucleotide (nt) 1−1min aptamer had a binding profile nearly identical to the larger 112 nt 1−1 aptamer and was therefore used for further analysis. The conserved stem loop (nts 37−55) alone showed no affinity for ATP.

To study the nature of the interaction of the 1−1min aptamer with the triphosphate moiety of ATP, we carried out solution binding studies by equilibrium ultrafiltration.[4] Binding of 1−1min to ATP in solution was highly dependent on [Mg^{2+}], with maximal binding at approximately 30 mM (Supporting Information Figure 1). Competition binding experiments with ATP, ADP, and AMP were performed at both high and low Mg^{2+} concentrations. Under the original selection conditions of 10 mM $MgCl_2$, the K_d of 1−1min for ATP and AMP was 11 and 1700 μM, respectively, a difference of ∼150-fold (Figure 3A). However, at 30 mM $MgCl_2$, the K_d values for ATP, ADP, and AMP were 4.8, 310, and 5500 μM, respectively. For AMP versus ATP, the difference was 1100-fold (Figure 3B), corresponding to a $\Delta\Delta G$ of binding of 4.3 kcal/mol. The above data show that the 1−1min aptamer interacts specifically with the β and γ phosphate groups of ATP. Furthermore, as [Mg^{2+}] increases, the aptamer binds AMP less and ATP more tightly, indicating a crucial role for Mg^{2+} in recognizing the triphosphate.

The binding specificity of the 1−1min aptamer was further characterized with a series of NTPs and other ATP analogues (Figure 4). Unlike previous ATP aptamers,[6,7] 1−1min showed observable binding to GTP, UTP, and CTP. Removing either the 2′ or 3′ hydroxyl group from ATP caused an ∼10-fold reduction in affinity, and changes to the hydrogen bond donors and acceptors of the nucleobase also decreased affinity. Along with CTP and UTP, GTP and ITP suffer the greatest reductions in affinity, suggesting that in addition to possible stacking interactions, the 6-amino group of adenine is a significant point of contact to the 1−1min aptamer. However, these effects are small compared to the effect of removing the β and γ phosphates, emphasizing the importance of triphosphate recognition to the overall affinity of this aptamer to its target.

```
          ..((((((....))))))AG--((((((....))))))--((((((AGGAG))))))..
1-1MIN    GGGAGAUCUACGGAUCUCAG--GGCUCUUACGGGAGCU--ACAUGGAAGGAGUCCAUGU..
1-1       GGGAGAUC....GAUCUCAG--GGCUCU....GGAGCU--ACAUGGAAGGAGUCCAUGU..
1-2       GGGAG..........CUCAG--GU----.........-----AU.-GCAGGGAAGGAGUCCCUGC..
1-3       GGGAGAUC....UAUCUCAG--GGA---.........---UUC.-UUGCAGAAGGAGUCUGCAA..
1-4       GGGAGAUC....GAUAUCAG..GACUCU....AGGGUU.-ACCAGGAAGGAGUCCUGGU..
1-5       GGGAGAUC....UGUCUCAG..CC.CGG....CCG-GG.-AUUCGGAAGGAGUCCGAAU..
1-6       GGGAGAUC....GAUCUCAG--GUGACU....AGUCAC.-CCUUGGAAGGAGUCCAAGG..
1-7       GGGAGAUC....GUUCUCAG----------.....-------.-AGCGGGAAGGAGCCCUGCU..
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Figure 1. Sequence alignment of class 1 aptamers. Base-paired stems are indicated by parentheses; conserved nucleotides are colored. Dashes are place holders for alignment; dots indicate nonconserved nucleotides.

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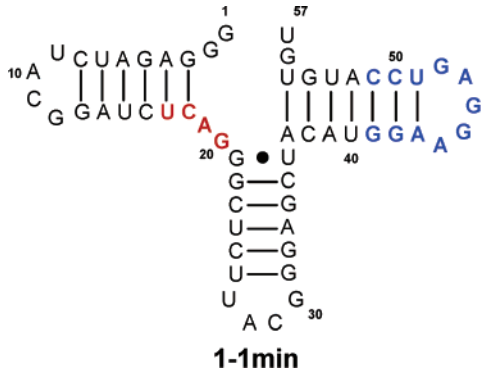

Figure 2. Secondary structure of the 1−1min aptamer. The class 1 conserved stem loop and linkage between two stem loops are highlighted in blue and red, respectively.

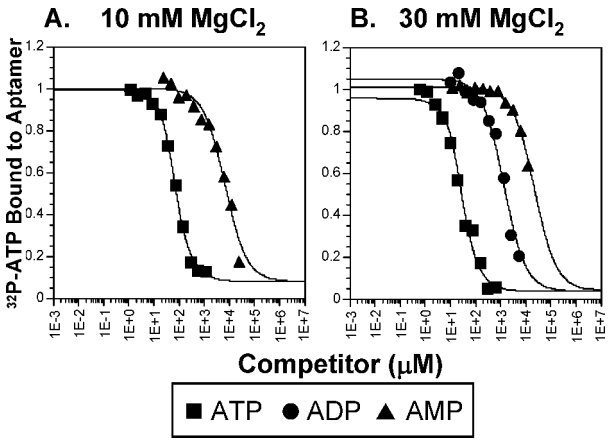

Figure 3. Binding of the 1−1min aptamer to ATP, ADP, and AMP. (a) For each data point, 35 μM 1−1min aptamer was incubated with 0.5 nM γ-^{32}P labeled ATP for 4 h in buffer containing 10 mM $MgCl_2$. (b) For each data point, 15 μM 1−1min aptamer was treated as in (a) with 30 mM $MgCl_2$ containing buffer. For both (a) and (b), the fraction of labeled ATP bound to the aptamer was determined and plotted against unlabeled competitor concentration.

Replacement of the $\beta-\gamma$ bridging oxygen with nitrogen caused a small reduction in affinity compared to ATP, while a methylene substitution had little effect. Interestingly, methylene substitution for the $\alpha-\beta$ bridging oxygen had a stronger effect, reducing affinity to that of ADP. Magnesium coordination of the phosphates is not affected significantly by these modifications.[13−15] However, it is possible that the $\alpha-\beta$ methylene group repositions the β and γ phosphates relative to the rest of the molecule such that the γ phosphate becomes unrecognizable by the 1−1min aptamer.

These results demonstrate that the 1−1min aptamer is capable of both strong and highly specific interactions with a triphosphate group in the presence of Mg^{2+}. The strong Mg^{2+} dependence of this recognition suggests that it is mediated by Mg^{2+} bridging interactions between the negatively charged RNA and the phosphates of ATP. Despite the presence of similar Mg^{2+} concentrations, such significant recognition of the triphosphate has not been observed in previous selections for nucleotide aptamers.[5−8]

The class 1 aptamer binds only slightly less strongly to ATP than aptamers[6,7] isolated from similar random-sequence RNA pools and selection protocols; why has it not been seen before? The aptamers that recognize ATP solely through interactions with its

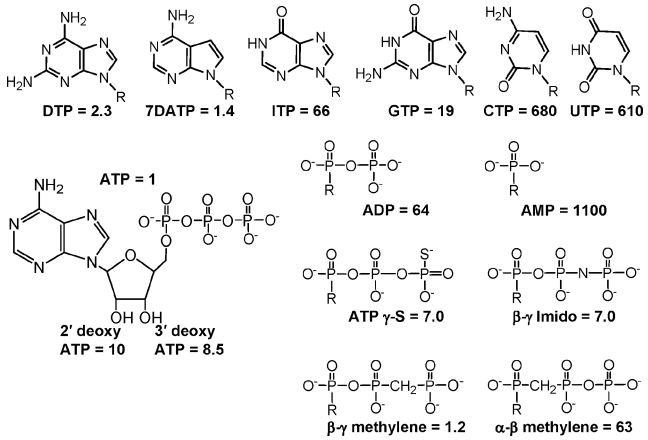

Figure 4. Analysis of 1−1min binding to nucleotides and analogues. Experiments were done as described in Figure 3B, but with the indicated analogue. The numbers indicate the K_d value relative to ATP (ATP = 1). ITP, inosine triphosphate; DTP, 2,6-diaminopurine triphosphate; 7DATP, 7-deazaadenosine triphosphate.

adenosine moiety seem to have less complex secondary structures than 1−1min, which makes them much more abundant in random sequence space. The class 1 aptamer was selected only after first selecting against those simpler sequences. Therefore, increased complexity is required for an aptamer to achieve phosphate discrimination, but this study demonstrates that the task can still be accomplished by relatively small sequences. The complexity of the *E. coli* riboswitches may be related to the several tasks that these leader sequences must perform, but it does not reflect an intrinsic limitation of the ability of RNA to interact with anionic targets.

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Supporting Information Available: Experimental detail and additional results. This material is available free of charge via the Internet at http://pubs.acs.org.

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