Designed Signaling Aptamers that Transduce Molecular Recognition to Changes in Fluorescence Intensity

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Abstract: We have engineered aptamers that contain fluorescent reporters and that signal the presence of cognate ligands in solution. Two different anti-adenosine "signaling aptamers", one made from RNA and one from DNA, can selectively signal the presence of adenosine in solution. Increases in fluorescence intensity reproducibly follow increases in adenosine concentration, and can be used for quantitation. The facile methods we have developed can potentially be used for generating a wide variety of signaling aptamers for use in sensor arrays.

Introduction

Most conventional diagnostic assays rely on the immobilization of either biopolymer receptors or their ligands. Such assays tend to be time-consuming and labor-intensive, and there is thus a pressing need for the development of homogeneous assay formats that do not require multiple immobilization or washing steps. For example, Gilardi et al. have conjugated fluorescent dyes to maltose-binding protein and were able to directly read maltose concentrations in solution,[1] and Marvin and Hellinga have conjugated fluorescent dyes to glucose-binding protein and followed glucose concentrations in solution.[2] Oligonucleotides and nucleic acids have previously been adapted to sense hybridization[3] and could potentially be used to detect metals.[4] We have now adapted selected nucleic acid binding species (aptamers) to similarly signal the presence of cognate analytes.

The conversion of ligand-binding proteins[5] or small molecules[6] to biosensors is highly dependent on the structure and dynamics of a given receptor, and it may be simpler to convert aptamers to biosensors[7,8] for two important reasons. First, aptamers by and large seem to undergo an "induced fit" conformational change in the presence of their cognate ligands,[9] and thus an appended dye might easily undergo a ligand-dependent change in its local environment. Second, in contrast to other reagents, such as antibodies, aptamers can be readily synthesized and dyes can be easily introduced into specific sites. Thus, aptamer biosensors can potentially be quickly generated using both rational and random engineering strategies.

There have been several published three-dimensional structures of aptamers that bind small, organic ligands.[10−14] We have used the structures of two anti-adenosine aptamers,[11,12,15] one selected from an RNA pool[16] and one selected from a DNA pool,[17] as starting points for the design of "signaling aptamers" (Figure 1). The program Insight 2 (Molecular Simulations) was used to visualize and manipulate the structures of these anti-ATP aptamers. Fluorescent dyes were placed adjacent to functional residues, and the signaling abilities of the resultant chimeras were evaluated by determining whether changes in fluorescence intensity occurred in the presence of the cognate ligand, ATP.

Results and Discussion

In initial models, fluorophores were either placed in proximity to the ligand-binding sites of aptamers, so as to avoid blocking or disrupting them, or were placed so that larger, ligand-induced conformational changes in aptamer structure (e.g., helical rotation) might be monitored. For example, residue 13 of the anti-adenosine RNA aptamer is adjacent to the binding pocket but does not participate in interactions with ATP and instead points outward, into solution (Figure 1a). An acridine moiety

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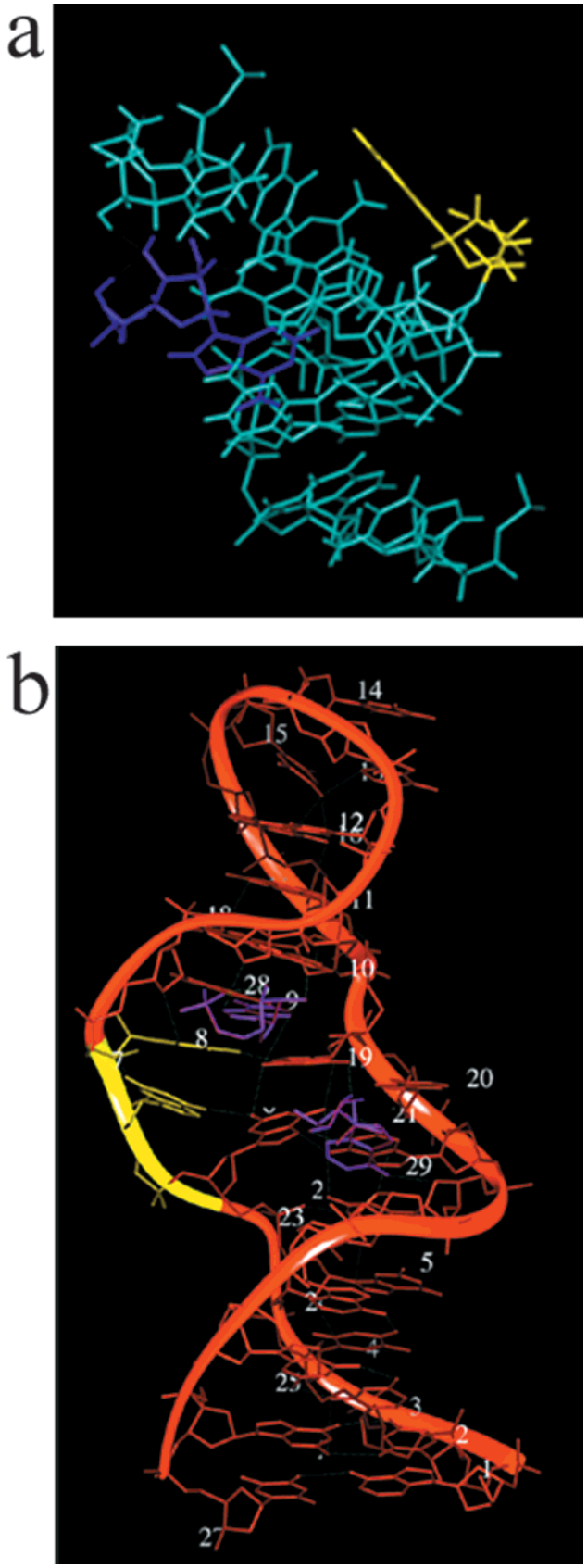

Figure 1. Three-dimensional models of anti-adenosine aptamers derived from NMR analysis.[11,12] Examples of some of the sites chosen for dye incorporation into either RNA (blue; ATP-R-Ac13) or DNA (orange; DFL7-8) aptamers are shown in yellow. Bound adenosines are shown in purple.

was therefore introduced into the RNA aptamer in place of the adenosine at position 13 (ATP-R-Ac13; Figure 2). Similarly,

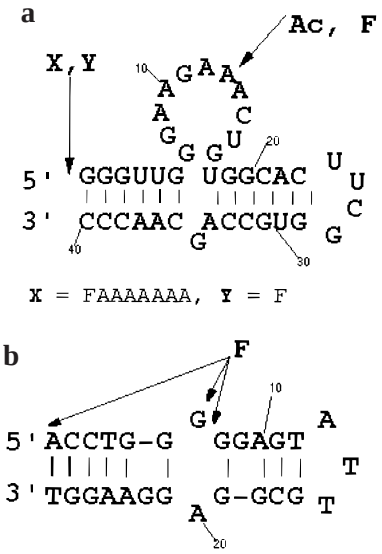

Figure 2. Sites of dye incorporation into aptamers. (a) RNA aptamers: Acridine was incorporated in place of residue 13 (ATP-R-Ac13). Fluorescein was incorporated at the 5′ end (ATP-R−F1), at the 5′ end with a heptaadenyl linker (ATP-R-F2), and in place of residue 13 (ATP-R-F13). (b) DNA aptamers: Fluorescein was incorporated at the 5′ end (DFL0), in place of residue 7 (DFL7), and between residues 7 and 8 (DFL7-8). Residues are numbered from the 5′ end on the secondary structures.

residue 7 in the DNA aptamer is in proximity of the binding site, and has no direct interactions with ATP (Figure 1b), and therefore fluorophores were placed in place of residue 7 and between residues 7 and 8 (DFL-7 and DFL7-8; Figure 2).

Of the various constructs tested, the ATP-R-F1, ATP-R-F2, ATP-R-F13, DFL0, and DFL7 aptamers showed an insignificant change in fluorescence intensity (5% or less) upon the addition of ATP. However, the ATP-R-Ac13 and DFL7-8 aptamers showed marked increases in fluorescence intensity in the presence of 1 mM ATP. The increases in response ranged from 25 to 45%.

To assess the specificity of these two signaling aptamers for ATP, changes in fluorescence were also measured in the presence of GTP, CTP, and UTP (Figure 3). No significant, ligand-dependent increases in fluorescence were observed. In addition, mutant versions of ATP-R-Ac13 and DFL7-8 that did not bind to ATP were constructed by omitting or replacing key functional residues. Residue G34 of the RNA aptamer is known from mutagenesis studies to be essential for binding,[16] while residues G9 and G22 in the DNA aptamer are critical contacts for the ATP ligands. We therefore constructed a mutant of the RNA aptamer lacking G34 (Mut 34) and a double mutant of the DNA aptamer in which both G9 and G22 were replaced with cytidine residues (Mut 9/22). As expected, the mutant signaling aptamers showed no ATP-dependent increases in fluorescence (Figure 4).

To determine if signaling aptamers could be used to quantitate analytes in solution, response curves were obtained by measuring the fluorescence intensities of ATP-R-Ac13 and DFL7-8 as a function of ATP and GTP concentrations (Figure 5). Both signaling aptamers showed a graded increase in fluorescence intensity with ATP, but little or no change in fluorescence intensity with GTP. While the response curves for the signaling aptamers were completely reproducible they could not be fit by simple binding models based on the reported K_d values of the original aptamers. However, the original binding data for the DNA aptamer[17] were based on the assumption that it

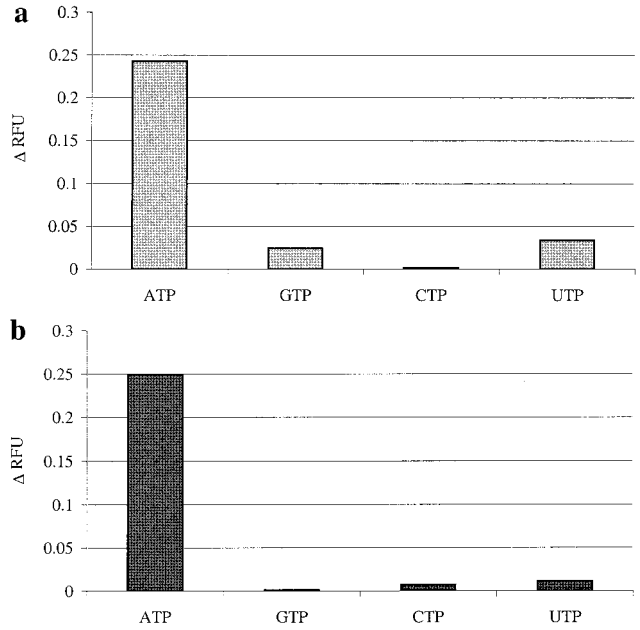

Figure 3. Specificities of the signaling aptamers ATP-R-Ac13 (a) and DFL7-8 (b). The fractional increase in relative fluorescence units (ΔRFU) was measured in the presence of ATP, GTP, CTP, and UTP (1 mM ligand for ATP-R-Ac13, 200 μM ligand for DFL7-8).

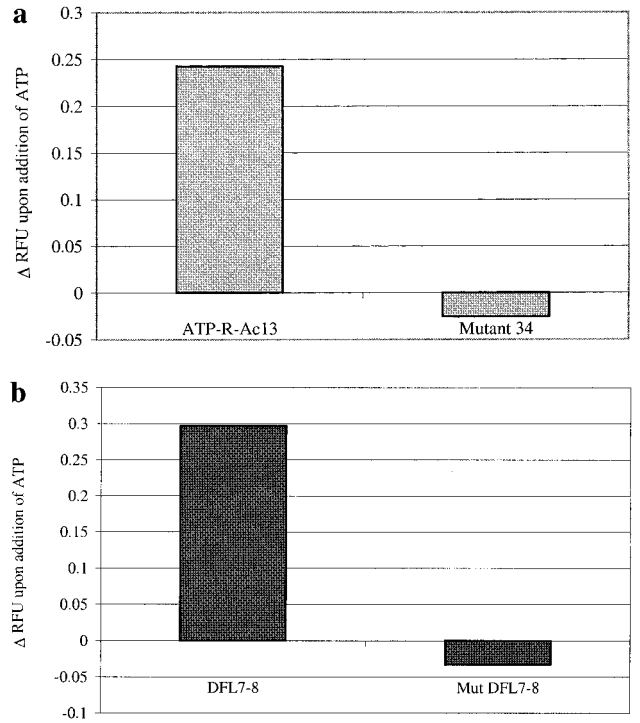

Figure 4. Mutant versions of signaling aptamers ATP-R-Ac13 (a) and DFL7-8 (b) do not signal. The ΔRFU was measured in the presence of ATP (1 mM ligand for ATP-R-Ac13 and Mut34, 250 μM ligand for DFL7-8 and Mut9/22).

contained only a single ligand-binding site, while the NMR structure had revealed two ligand-binding sites. To determine whether the signaling aptamer might be detecting both ATP-binding sites, the change in fluorescence was plotted against the ratio of the change in fluorescence to the concentration of unbound ATP. The resulting nonlinear Scatchard plot (Figure 6) was biphasic, suggesting that multiple binding sites were perceived. We attempted to fit the signaling data to a model in which the aptamer cooperatively binds to two ATP molecules,

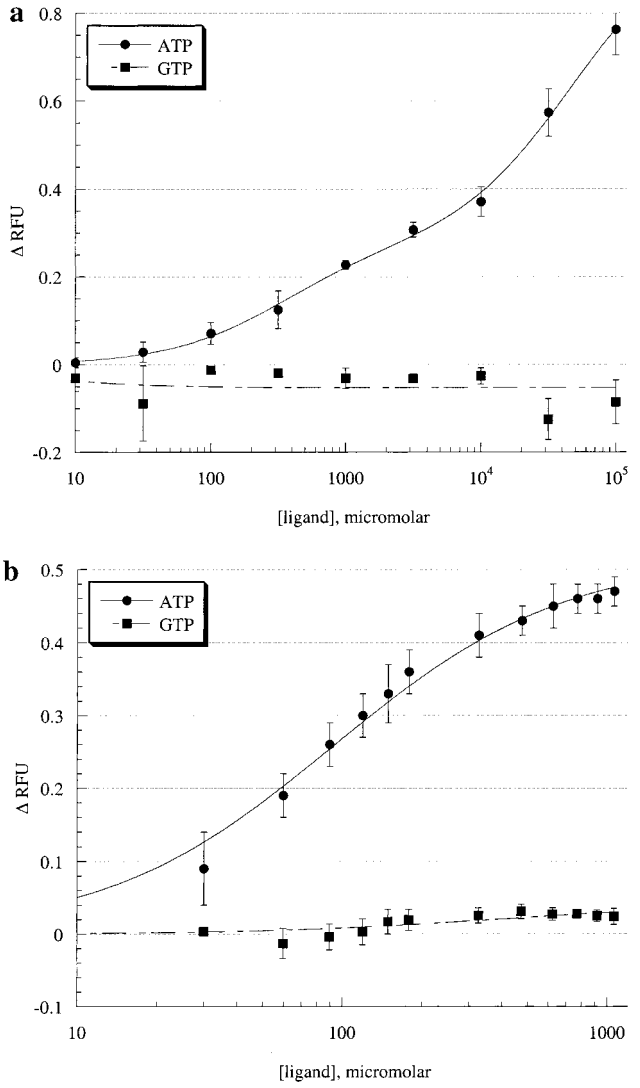

Figure 5. Response curves for the signaling aptamers ATP-R-Ac13 (a) and DFL7-8 (b). The ΔRFU plotted at various concentrations of ATP (●) and GTP (■). Data points are shown as an average of three values with standard deviations. Data were curve-fitted using the program Kaleidograph (Synergy Software).

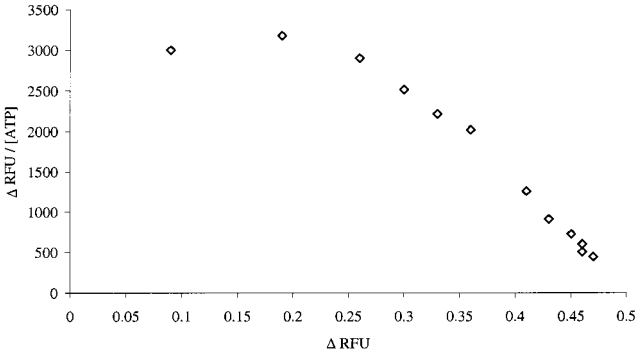

Figure 6. Scatchard plot derived from the response curve of the DNA signaling aptamer. The fractional increase in RFU (ΔRFU; *x* axis) is plotted against the ratio of ΔRFU/[ATP] (*y* axis).

using the following equation:

$$(F - F_0) = \frac{K_1(F_1 - F_0)[\mathrm{L}] + K_1K_2(F_2 - F_0)[\mathrm{L}]^2}{1 + K_1[\mathrm{L}] + K_1K_2[\mathrm{L}]^2}$$

where F is the fluorescent signal, F_0 is the fluorescence of

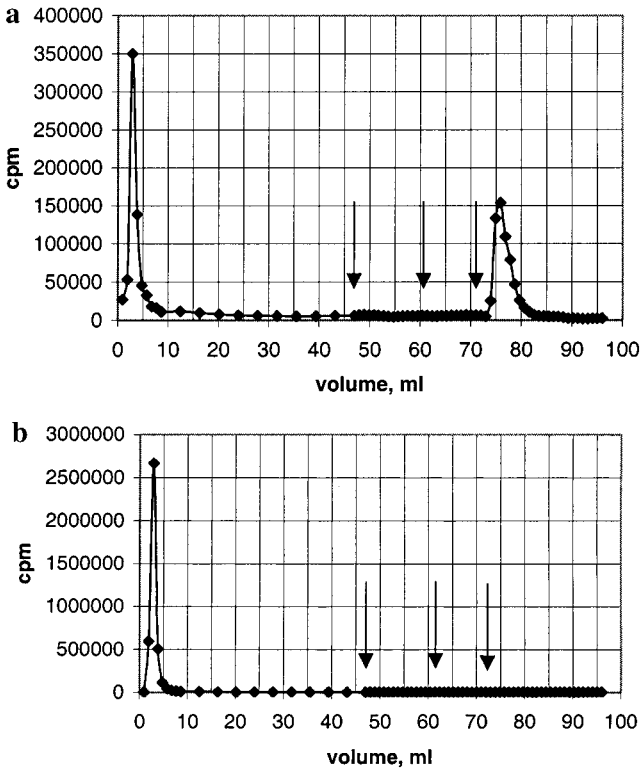

Figure 7. Elution profiles for the signaling aptamer DFL7-8 (a) and its double mutant Mut9/22 (b). After applying the radiolabled aptamer, the column was washed with 44 mL of selection buffer. A 0.3 mM GTP solution in selection buffer (15 mL) was applied (first arrow from left). After washing the column with an additional 10 mL of selection buffer (second arrow), a 0.3 mM ATP solution in selection buffer (15 mL) was added (third arrow).

uncomplexed substrate, F_1 is the fluorescence of singly bound substrate, F_2 is the fluorescence of doubly bound substrate, K_1 is the formation constant of first-order complex, and K_2 is the formation constant of second-order complex

This analysis yielded two dissociation constants, indicating a higher affinity site with a $K_{d,1}$ ($1/K_1$) of 30 ± 18 μM, and a lower affinity site with a $K_{d,2}$ ($1/K_2$) of 53 ± 30 μM. The relative change in fluorescence upon binding first ATP (F_1) is calculated to be negligible, -0.004%, while the relative change in fluorescence due to the formation of the ternary complex (F_2) is calculated to be 49%. The similarity in affinity between the two binding sites is consistent with the sequence and structural symmetry of the DNA, anti-adenosine aptamer. Given that the greatest change in fluorescence is observed upon ternary complex formation, it can be hypothesized that the affinity of the site containing the fluorescein reporter has been slightly perturbed and that the signaling aptamer is primarily reporting ligand interactions with this site.

To independently examine the binding abilities of the signaling aptamers, we used an isocratic elution technique that had previously been used to determine aptamer K_d values for ATP.[16] In short, the signaling aptamers were applied to an ATP affinity column identical with that used in the original selection experiments and were progressively eluted with buffer and nucleotides. The RNA signaling aptamer ATP-R-Ac13 bound poorly to the column; its estimated K_d is greater than millimolar. These results accord with the relatively large amounts of ATP required to generate a signal (Figure 5a). The diminution in the affinity of the RNA aptamer upon the introduction of acridine is similar to diminutions in affinity observed upon the introduction of dyes into maltose- and glucose-binding proteins.[1,2] In contrast, the DNA signaling aptamer DFL7-8 (Figure 6a) had an apparent K_d that was lower than 13 μM, and could not be eluted from the ATP affinity column with GTP. The affinity of the DNA aptamer inferred from column chromatography is comparable to the calculated affinity of the lower affinity site, above. The nonsignaling double mutant, Mut9/22, did not bind to the affinity column (Figure 6b). The lower K_d of the DNA signaling aptamer relative to the RNA signaling aptamer accords with a better signaling response by the DNA signaling aptamer (Figure 5b). However, it is difficult to directly compare binding and signaling studies with the DNA aptamer, since the unmodified aptamer contains two, cooperative adenosine binding sites[17] which may have been differentially affected by the introduction of the dye.

Aptamers have previously been introduced into diagnostic assays, although their primary use has been as substitutes for antibodies. The fact that aptamer−dye conjugates can directly signal the presence and amount of analytes in solution without the need for prior immobilization or washing steps may allow aptamers to be used in ways that are currently unavailable to antibodies. Since aptamers have already been selected against a wide array of target analytes, from ions to small organics to proteins to supramolecular structures such as viruses or tissues,[18,19] it may prove possible to quickly synthesize numerous, new reagents for sensor arrays by the simple addition of fluorescent dyes to extant aptamers, as described here. The fact that the first generation of designed compounds can detect analytes in the micromolar to millimolar range makes this possibility even more likely. The sensitivity of signaling aptamers can likely be further refined by exploring the incorporation of a wider range of dyes at a wider range of positions (for example, while the acridine-containing conjugate ATP-R-Ac13 signaled quite well, the fluorescein-containing conjugate ATP-R-F13 signaled poorly), by using multiple dyes in FRET or quenching strategies, and by the inclusion of fluorescent dye−nucleotide conjugates during the selection experiments themselves. For example, pools containing a limited mole fraction of a given nucleotide could be synthesized and replicated or transcribed with a fluorescent nucleotide analogue. Following selection for binding, individual aptamers could be screened for their ability to signal the presence of a cognate ligand. Any signaling aptamers generated by this method would presumably either utilize or accommodate fluorescent reporters during ligand binding.

Experimental Section

Materials. ATP (disodium salt) and GTP (disodium salt) were purchased from Roche Molecular Biochemicals, and ATP agarose (C8 linkage, 9 atom spacer) was purchased from Sigma. Fluorescein phosphoramidite, 5′-fluorescein phosphoramidite, and acridine phosphoramidite were purchased from Glen Research. T4 polynucleotide kinase and polynucleotide kinase buffer were purchased from New England Biolabs. Radioactive [γ-^{32}P] ATP was purchased from ICN.

Preparation of Signaling Aptamers. A series of aptamer−dye conjugates (Figure 2) were synthesized and deprotected as described previously.[20−23] Fluorescein phosphoramidite and acridine phosphoramidite were used in the syntheses of the internally labeled aptamers while the terminally labeled aptamers were generated using 5′-

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fluorescein phosphoramidite. Deprotection of the RNA aptamer−dye conjugates was carried out using a procedure modified from Wincott et al.[23] In the first part of the deprotection, the resins were suspended in 3:1 NH_4OH:EtOH for 13 h at room temperature, rather than for 17 h at 55 °C. The aptamers were purified by polyacrylamide gel-electrophoresis, eluted with 0.3 M NaOAc overnight at 37 °C, and ethanol precipitated. The aptamers were resuspended in 50 μL of H_2O and subsequently quantitated by measuring the A_{260} using an extinction coefficient of 0.025 mL cm^{-1} μg^{-1} for RNA and 0.027 mL cm^{-1} μg^{-1} for DNA.

The aptamers were thermally equilibrated in selection buffer and conditions were empirically determined to give the optimal fluorescence intensity. Before taking fluorescence measurements, the RNA aptamers (500 nM) were suspended in selection buffer, 300 mM NaCl, 20 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$,[16] heat denatured at 65 °C for 3 min, and then slow-cooled to 25 °C in a thermocycler at a rate of 1 deg C per 12 s. The DNA aptamers (150 nM) were suspended in selection buffer,[17] heat denatured at 75 °C for 3 min, and allowed to cool to room temperature over 10−15 min.

Fluorescence Measurements. All fluorescence measurements were taken on a Series 2 Luminescence Spectrometer from SLM-AMINCO Spectronic Instruments. The experimental samples were excited at their respective maximums (acridine, $\lambda_{ex} = 450$ nm; fluorescein, $\lambda_{ex} = 495$ nm) and fluorescence intensity was measured at the corresponding emission maximums (acridine, $\lambda_{em} = 495$ nm; fluorescein, $\lambda_{em} = 515$ nm). The aptamer solutions (200 μL for RNA, 1000 μL for DNA) were pipetted into a fluorimeter cell (Starna Cells, Inc.**)** and ligand solutions of varying concentrations but standard volumes (50 μL for RNA, 1.5 μL for DNA) were added.

Measurements of Binding Affinities by Isochratic Elution. For 5′ end-labeling, the aptamers were were incubated for 1 h at 37 °C in a T4 polynucleotide kinase reaction mix (1 μL T4 polynucleotide kinase (10 units), 2 μL DNA, 0.5 μL 10× polynucleotide kinase buffer, 0.5 μL [γ-^{32}P] ATP (7000 Ci/mmol), 6 μL H_2O for a total volume of 10 μL). A column of ATP agarose, with a total volume (V_t) of 1.5 mL and a void volume (V_o) of 1.16 mL, was equilibrated with 25 mL of selection buffer. Aptamers (10 μg) were thermally equilibrated and applied to the column. The concentration of ATP ([L], see below) on the column was 2.6 mM. The column was then washed with selection buffer and 1 mL fractions were collected. Portions (5 μL) of each fraction were spotted on a nylon filter and the amount of radioactivity present was quantitated with a Phosphorimager (Molecular Dynamics). The column was developed with an additional 44 mL of selection buffer, followed by 15 mL of a 0.3 mM GTP solution in selection buffer. After washing the column with an additional 10 mL of selection buffer, 15 mL of a 0.3 mM ATP solution in selection buffer was found to completely elute any remaining radioactivity. For the aptamer DFL7-8, a final elution volume (V_e) of 73 mL was used to develop the column prior to the addition of the ATP solution. An upper bound for the K_d of the signaling aptamer for ATP-agarose was calculated using the equation $K_d = [L](V_t - V_o)/(V_e - V_o)$.[16]

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