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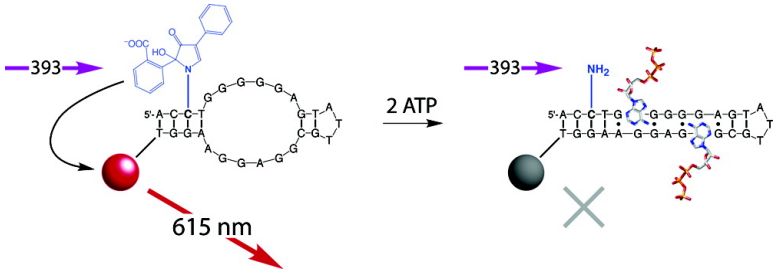

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Fluorogenic Resolution of Ligand Binding by a Nucleic Acid Aptamer

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Iterative in vitro selection and amplification can be used to identify small nucleic acid molecules, called aptamers, capable of binding specifically and with good-to-very high affinities to diverse target ligands.[1] Specific molecular recognition occurs via stacking and hydrogen-bonding interactions to the target and is typically accompanied by ligand-induced structural changes in both RNA[2] and DNA[2,3] aptamers. An important, but currently imperfectly realized, goal is to develop generic approaches for coupling small-molecule binding events to signal readout. We report a straightforward technology for detecting ligand binding by an aptamer that yields a useful permanent readout and solution-phase fluorescence detection.

The DNA-based ATP aptamer[4] binds two ATP molecules in a noncanonical, but stable, helix comprised of G:G and G:A base pairs flanked by short canonical helices[5] (Figure 1A). This ATP aptamer has been widely used as a benchmark for converting ligand binding into a suitable sensor readout.[6] Prior to ligand binding, the purine-purine pairs are not stably formed, and ATP recognition therefore involves induced fit, as is typical for DNA aptamers.[2,3]

The nucleophilic reactivity of a 2′-amine substituted nucleotide is gated by the underlying local flexibility in both RNA[7,8] and DNA[9] structures. 2′-Amine-substituted nucleotides in flexible positions are better able to adopt a high reactivity local structure in which the partial positive charge on the amine nucleophile in the transition state is stabilized by the adjacent 3′-phosphodiester anion[10] (see green dashed line in Figure 1B). 2′-Amine-substituted nucleotides can be constrained, and thus rendered unreactive, by base pairing[9] or other interactions[7,8] that limit local flexibility. In our ATP sensor, a unique 2′-amine-substituted nucleotide is placed in a position expected to be flexible in the ligand-free state but becomes constrained upon ATP binding due to the substantial ligand-induced conformational change (Figure 1A).

Fluorogenic compounds,[11] including fluorescamine, (FCM)[12] are potentially well suited for converting aptamers into small-molecule sensors via reaction at the 2′-amine. FCM reacts with aliphatic amines (Figure 1B) in milliseconds[13] to form a stable fluorescent pyrrolinone (absorbance and emission at 390 and ∼480 nm, respectively). FCM undergoes hydrolysis in parallel to form nonfluorescent hydrolysis products, effectively quenching the reagent within seconds.

Three analogues of the ATP aptamer were synthesized,[14] each containing a single 2′-amine substituted cytidine. The 2′-amine substitutions were incorporated at positions 2, 3, and 17 (Figure 1A) and are termed the 2C, 3C, and 17C aptamers, respectively. Both the unstructured binding pocket and the short flanking helices are flexible in the absence of ATP, making the 2′-amine reactive (Figure 1A). Upon binding 2 equiv of ATP, formation of the noncanonical helix is expected to lower the reactivity of the unique 2′-amine.

Aptamers were 5′-^{32}P labeled, treated with a final concentration of 450 μM FCM, and resolved by gel electrophoresis. Bulky FCM adducts electrophorese more slowly than does the free aptamer

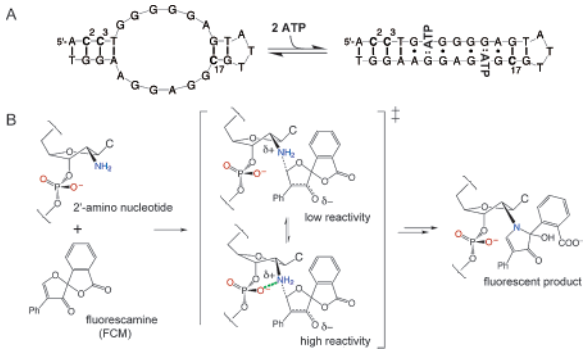

Figure 1. (A) Ligand-induced structural changes in the ATP aptamer. 2′-Amine-substituted nucleotides were introduced at each of three cytidine residues (in boldface). Upon binding 2 equiv of ATP, the aptamer forms a stable duplex incorporating noncanonical base pairs. (B) Reaction of fluorescamine with a 2′-amine to form a fluorescent adduct. The fluorogenic reaction is more efficient in flexible structures that more frequently populate the high reactivity transition state.

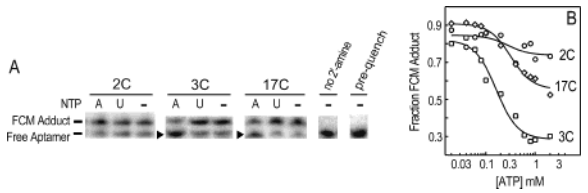

Figure 2. (A) Electrophoretic separation of free aptamers and low-mobility FCM adducts for the 2C, 3C, and 17C aptamers. ATP or UTP concentrations, if present, were 2 mM. Pre-quench reactions were performed by adding FCM prior to the 3C aptamer. (B) Apparent binding curves, assuming cooperative binding by two equivalents of ATP.[14]

(Figure 2A). In the absence of ATP, all three aptamers react to form 80−90% FCM adduct (see no, —, NTP lanes in Figure 2A). Formation of a FCM adduct requires a 2′-amino group because a DNA aptamer lacking the 2′-amine does not form a low-mobility product (no 2′-amine lane in Figure 2A). Addition of FCM prior to the 3C aptamer also does not yield a low-mobility adduct, confirming that FCM is rapidly inactivated by hydrolysis in the absence of amine. Upon addition of the cognate ATP ligand, reaction with FCM is substantially reduced for the 3C and 17C aptamers (Figure 2A, see arrows in the ATP lanes). The decrease in reactivity is specific for ATP because addition of UTP has no effect on FCM adduct formation (compare UTP to no nucleotide lanes in Figure 2A). When comparable experiments are performed as a function of ATP concentration, the data are well fit (squares and diamonds in Figure 2B), assuming protection from adduct formation reflects cooperative binding by 2 equiv of ATP. The 3C and 17C aptamers bind ATP with $K_{1/2}$ values of 160 and 570 μM, respectively. These binding constants are comparable to the 100−600 μM values typically obtained for ATP binding by this aptamer in solution.[6]

In contrast, FCM reactivity with the 2C aptamer was barely modulated by addition of ATP (circles in Figure 2B). This suggests

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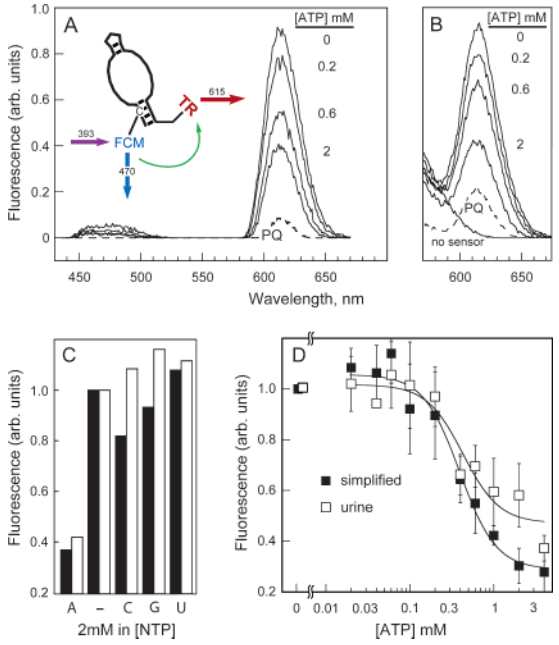

Figure 3. 3C-Texas red (3C-TR) sensor. Resonance energy transfer-based detection of nucleotide binding in a solution containing simplified components (A) or human urine (B). PQ, pre-quench, and no sensor indicate reactions in which FCM was added prior to the aptamer or aptamer was omitted. (C) Fluorogenic response of the aptamer to nucleotide triphosphates or no (−) added nucleotide. Solid and open bars indicate response in simplified or urine-containing solutions. Data are normalized to the no-nucleotide case. (D) Apparent ATP binding affinities for reactions performed in simplified or urine-containing solutions (closed and open squares, respectively). Error bars indicate standard deviations for experiments performed in triplicate.

that the local environment of a 2′-amine group at position 2 is largely governed by fraying of the duplex near its ends. Apparently, a 2′-amine group should be placed close to a structure that experiences induced fit, but at least three base pairs from a helix end.

The 3C aptamer showed the greatest difference in FCM reactivity upon binding by ATP (squares in Figure 2B). We therefore explored converting the 3C aptamer into a resonance energy transfer sensor. The 3C aptamer was synthesized with a 3′-Texas red fluorophore (see inset in Figure 3A).[14]

We used the 3C-Texas red (3C-TR) aptamer to detect ATP binding in solution. The 3C-TR aptamer was treated with FCM as a function of nucleotide concentration. FCM was selectively excited at 393 nm, and emission intensity was recorded from 425 to 670 nm (Figure 3A). We observe a small shoulder at 470 nm, corresponding to direct emission by FCM, and a large peak at 615 nm, reflecting energy transfer and subsequent emission by the Texas red fluor, via a very large red shift ($\Delta\lambda = 222$ nm). In contrast, reactions in which FCM was added (and decomposed) prior to the addition of the 3C-TR aptamer showed no FCM shoulder and a small peak at 615 nm due to partial direct TR excitation (see pre-quench, PQ, spectra in Figure 3A). Addition of ATP reduces the fluorescence intensity at 615 nm due to resonance energy transfer (Figure 3A) because the 2′-amine is constrained to a low reactivity state, thereby disfavoring formation of the FCM donor fluor (Figure 1B).

The 3C-TR sensor was then tested in a background of 33% (v/v) human urine.[14] Endogenous amines were first acylated with sulfosuccinimidyl acetate, and added nucleotides were subsequently detected by the fluorogenic reaction with FCM. ATP is detected in the urine solution with a sensitivity comparable to detection in the simple buffer solution (Figure 3B). Detection is robust even though there is a very large background signal between 400 and 570 nm due to the large intrinsic fluorescence typical of biological samples and to residual reactivity of solution amines (see no sensor reaction, Figure 3B).

Addition of the noncognate CTP, GTP, and UTP ligands has little or no effect on the observed fluorescence signal for sensor reactions performed in both simplified and urine-containing solutions (closed and open bars, respectively, in Figure 3C). Monitoring fluorescence intensity at 615 nm as a function of ATP concentration yields essentially identical $K_{1/2}$ values of 390 and 430 ($\pm$70) μM for the 3C-TR aptamer in the simplified and urine solutions, respectively (closed and open squares, Figure 3D).

We have taken advantage of the differential reactivity of 2′-amine substituted nucleotides in flexible versus constrained nucleic acid structures (Figure 1) to chemically resolve bound and ligand-free states of an ATP aptamer in both simple and biologically complex solutions. The ligand-gated reaction with FCM is robustly detected via resonance energy transfer to an appropriate fluor and yields a permanent record of ligand binding. In addition, creating small-molecule sensors by this approach requires very little information about the aptamer: only identification of a local ligand-induced conformational change. Identification of an appropriate site for the 2′-amine substitution is straightforward, even when the underlying molecular details are unknown,[8] and may be achieved for large-scale aptamer applications using combinatorial approaches[7] or secondary structure-based rational design. This selective fluorogenesis chemistry should prove useful for creating sensors from diverse aptamers that bind small molecule ligands via induced-fit.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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