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Detection of Small Organic Analytes by Fluorescing Molecular Switches

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Abstract—A sensor system was developed for the determination of theophylline concentrations based on a theophylline-dependent allosteric ribozyme (Soukup, G. A.; Breaker, R. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999,** 96, 3584) in combination with an RNA substrate which is double-labeled with a fluorophore and a quencher dye. In the presence of theophylline, a hammerhead ribozyme domain is switched into an active conformation by the action of a theophylline-binding aptamer domain. Upon substrate cleavage, the quencher is removed from the vicinity of the fluorophore, causing an increased fluorescence signal. Real-time analysis of the cleavage reactions both under single and multiple turnover conditions revealed a dependence on the cleavage rate within a range from 0.01 to 2 mM theophylline. The structurally similar molecule caffeine, however, had no detectable influence on the fluorescence signal.

Introduction

RNA molecules are known to precisely recognize their target molecules, and combinatorial techniques have successfully been used to isolate a wide variety of target-binding RNAs (aptamers).[1–3] On the other hand, RNA can possess enzymatic activity, and the naturally occurring ribozymes cleave complementary substrate RNAs with a well-understood, predictable specificity.[4,5] Recently, a combination of these features has emerged in the form of 'allosteric ribozymes' or 'molecular switches', in which the binding of an effector molecule modulates the activity of the ribozyme (Fig. 1a).[6–9] These molecules can be either rationally designed by assembling known aptamer and ribozyme domains, or they can be selected from combinatorial RNA libraries.[10,11] In addition to the academic value of demonstrating allostery for nucleic acid molecules, these molecular switches can be potentially useful for the detection and quantification of analytes.

Fluorescence resonance energy transfer (FRET) has been demonstrated a valuable technique for nucleic acid structure determination[12–14] and analysis of ribozyme reaction kinetics.[15–18] An important variant of this technique are the 'molecular beacons' (Fig. 1b) which allow for the specific detection of DNA or RNA sequences. The molecular beacon is a short oligonucleotide double-labeled with a fluorophore and a quencher dye. In the absence of the target sequence, it forms a hairpin structure in which the quencher resides

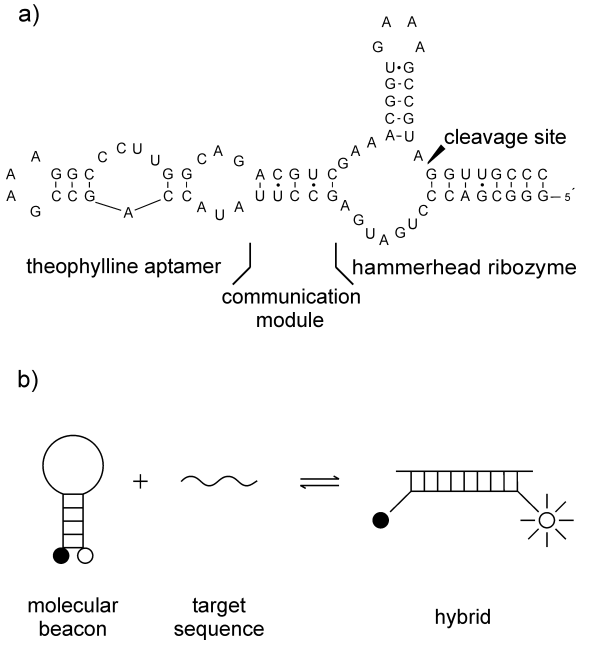

Figure 1. (a) Theophylline-dependent allosteric ribozyme[6] and (b) operating principle of molecular beacons.

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in the direct vicinity of the fluorophore, and fluorescence is efficiently quenched. Upon binding to the target sequence, the stem is opened, and an increased fluorescence signal can be measured, as the fluorescence intensity is highly dependent on the distance between the two dyes. Various applications of molecular beacons for real-time monitoring of nucleic acid synthesis and localization of specific RNA have been described.[19–22] Recently, double-labeled substrate oligonucleotides were used for real-time kinetic determination of ribozyme kinetics.[15–18]

We have now combined these two principles and report here allosteric ribozymes in combination with double-labeled substrate oligonucleotides for the determination of various analytes. The principle is demonstrated for the quantification of theophylline, a clinically relevant bronchiodilator.

Results and Discussion

Design of the sensor system

For the proof-of-principle experiments described here, we used an allosteric ribozyme originally developed by Soukup and Breaker (Fig. 1a).[6] It consists of the theophylline aptamer domain which is connected to the hammerhead ribozyme via a 4 bp communication module. In the absence of theophylline, no significant cleavage reaction occurs. Upon binding of theophylline a structural reorganization of the aptamer domain forces the communication module to switch and the conformation of the hammerhead ribozyme domain to become active. An allosteric regulation by a factor of 100 was reported.

While this system was designed for intramolecular hammerhead cleavage and detection by gel electrophoresis, we chose an intermolecular system allowing the investigation under both single and multiple turnover conditions (Fig. 2).

The overall structure with the three domains is identical to the original system except for the bimolecular nature of the system. It consists of a 58 nucleotide aptamer/ribozyme molecule (SWI 58) and a substrate oligonucleotide (SWI 13 DF). The substrate is 13 nucleotides long, carries a GUC hammerhead cleavage site, and has a low G/C content to facilitate product dissociation upon cleavage. Fluorescein and 4-[4′-Dimethylamino-phenylazo-] benzoic acid (Dabcyl) were used as end-labels, because they were reported to be a well-suited fluorophore-quencher pair.[21]

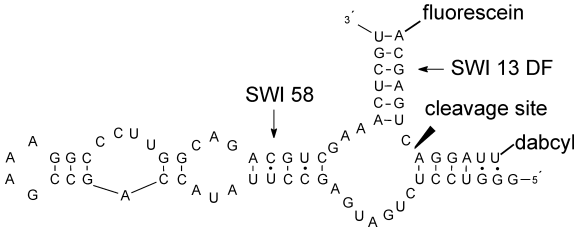

Figure 2. Theophylline-dependent allosteric ribozyme: intermolecular system SWI58/SWI13DF.

Theophylline-dependent substrate cleavage

The ribozyme-mediated cleavage reaction was analyzed in the presence of varying concentrations of theophylline under substrate or ribozyme excess. The relative cleavage rates were determined from the slope of the fluorescence versus time curves and could be correlated to the actual theophylline concentrations.

Figure 3a shows the fluorescence curves under conditions of ribozyme excess (single turnover), while the experiments in Figure 3b were done under multiple turnover conditions (excess of substrate oligonucleotide). In both systems, an increase in the theophylline concentration causes a faster development of a fluorescence signal. In the absence of theophylline, however, significant substrate cleavage occurred, too, which was much faster than that in the intramolecular system assay by gel electrophoresis. In the absence of theophylline, an increase of 0.08 relative fluorescence units per second (rFU/s) (ribozyme excess) and 0.11 rFU/s (substrate excess) was measured, while fluorescence increased at a rate of 0.3 rFU/s at 1 mM theophylline (ribozyme

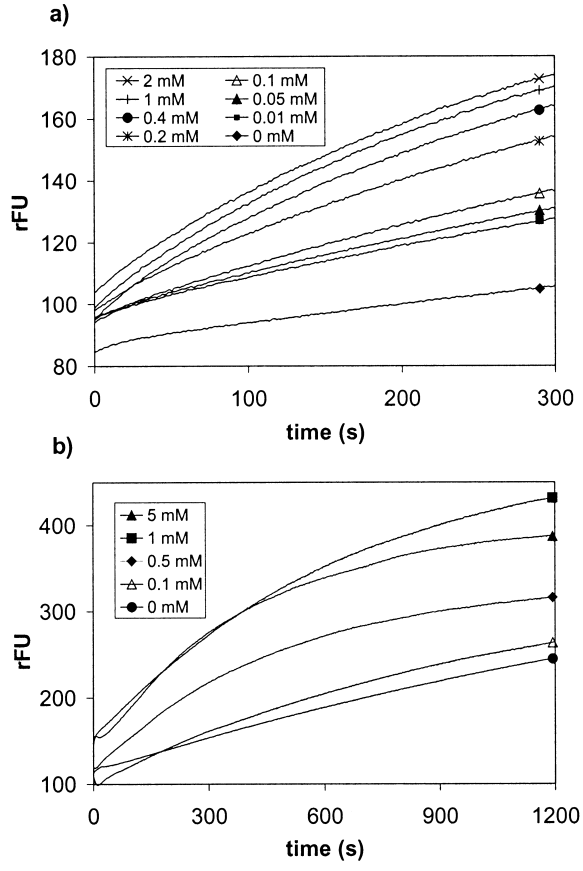

Figure 3. Time-resolved fluorescence signals of theophylline-mediated hammerhead cleavage in the system SWI58/SWI13DF (0–5 mM theophylline); (a) ribozyme excess; (b) substrate excess, (excitation 490 nm, emission 518 nm; rFU: relative fluorescence unit).

excess) and at 0.5 rFU/s with 5 mM theophylline (substrate excess). Half maximal time-dependent fluorescence increase is achieved at approximately 200 μM theophylline.

Relative cleavage rates could be correlated to the theophylline concentration (Fig. 4). The dynamic range of the sensor system lies in the range of 0.01–1 mM theophylline under ribozyme excess condition and 0.1–2 mM theophylline at substrate excess.

The allosteric modulation reported the intramolecular system of factor 100 could not be obtained.[6] Our results in the intermolecular system show only a 4-fold allosteric activation in the presence of theophylline.

Substrate cleavage in the presence of caffeine

An important criterion for the utility of a sensor system is its specificity for the analyte. The precise determination of theophylline in clinical samples is often complicated by the presence of caffeine, which differs from theophylline only by one methyl group. The theophylline aptamer was specifically developed to distinguish between these two molecules, and it binds caffeine 10,000-fold weaker than theophylline.[23]

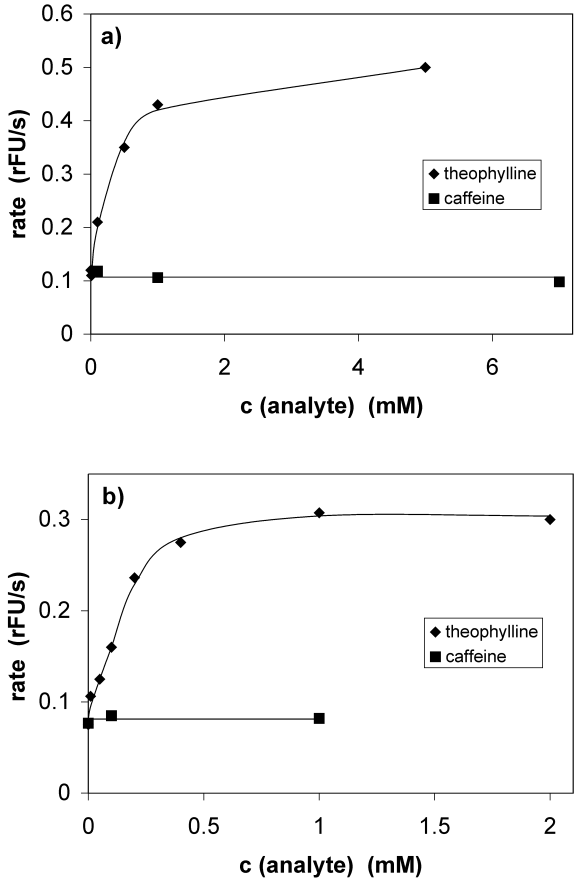

Figure 4. Relative cleavage rates versus concentration of analytes under conditions of (a) substrate excess; (b) ribozyme excess (rFU: relative fluorescence unit).

The influence of caffeine on the substrate-cleavage reaction was studied in the concentration range of 0.1–7 mM in the absence of theophylline (Fig. 4). No caffeine-dependent increase in the apparent cleavage rate could be measured, indicating that the theophylline selectivity of the aptamer part is retained.

Conclusion

In this work a dynamic system was developed for the direct and non-radioactive detection of theophylline on the basis of the hammerhead ribozyme and the theophylline aptamer. The time-resolved kinetic analysis of the theophylline dependent ribozyme activity could be performed by using the 'molecular beacon' technology. Theophylline could be detected in concentrations of 0.01–2 mM and caffeine did not influence the system.

The combination of allosteric ribozymes with fluorescence spectroscopy should in principle allow the construction of detection systems for a wide variety of analytes, ranging from metal ions over amino acids, antibiotics to proteins and even whole cells. Given the modular structure of the allosteric ribozymes, aptamers against these targets which are already available could be fused to a ribozyme via a communication module and combined with double-labeled substrates.

Experimental

The 58nt ribozyme strand SWI58 (5′-GGG UCC UCU GAU GAG CCU UAU ACC AGC CGA AAG GCC CUU GGC AGA CGU CGA AAC UCG U-3′) was synthesized by automated solid phase synthesis on a 8909 Expedite DNA/RNA synthesizer in a 1 μmol scale, deprotected and HPLC purified. The 13nt double end labeled substrate strand SWI13DF (5′-Fluorescein-ACG AGU CAG GAU U-Dabcyl-3′) was purchased from IBA (Göttingen) and PAGE purified.

The kinetics of the hammerhead cleavage rates were monitored by fluorescence measurements on a fluorescence/luminescence spectrometer (Perkin Elmer LS-50B). Fluorescence measurements were performed at an excitation wavelength of 490 nm and the emission was monitored at 518 nm.

For the measurements at substrate excess a solution of 50 mM HEPES-NaOH pH 7.6, 200 mM KCl, 1 mM EDTA, 25 mM $MgCl_2$, 1.72 μM SWI58 and varying theophylline or caffeine concentrations were prepared and transferred to a quartz cuvette (60 μL, Hellma). The recording of the time-course of the fluorescence signal was started after addition of SWI13DF (final concentration 7 μM) and followed for 10–30 min (slit excitation 15 nm, emission 5 nm).

For the measurements at ribozyme excess, a solution containing 100 mM Tris–HCl pH 7.6, 10 mM $MgCl_2$, 3.45 μM SWI58 and varying theophylline or caffeine concentrations were prepared and transferred to a

quartz cuvette (60 μL, Hellma). The recording of the time course of the fluorescence signal was started after addition of SWI13DF (final concentration 1.14 μM) and followed for 5 min (slit excitation 15 nm, emission 10 nm).

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