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Assay for Glucosamine 6-Phosphate Using a Ligand-Activated Ribozyme with Fluorescence Resonance Energy Transfer or CE-Laser-Induced Fluorescence Detection

Jennifer R. W. Furchak,† Peilin Yang, Colin Jennings, Nils G. Walter,* and Robert T. Kennedy*

Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, Michigan 48109-1055

A naturally occurring aptazyme, the *glmS* ribozyme, is adapted to an assay for glucosamine 6-phosphate, an effector molecule for the aptazyme. In the assay, binding of analyte allosterically activates aptazyme to cleave a fluorescently labeled oligonucleotide substrate. The extent of reaction, and hence analyte concentration, is detected by either fluorescence resonance energy transfer (FRET) or capillary electrophoresis with laser-induced fluorescence (CE-LIF). With FRET, assay signal is the rate of increase in FRET in presence of analyte. With CE-LIF, the assay signal is the peak height of cleavage product formed after a fixed incubation time. The assay has a linear response up to 100 (CE-LIF) or 500 μM (FRET) and detection limit of ~500 nM for glucosamine 6-phosphate under single-turnover conditions. When substrate is present in excess of the aptazyme, it is possible to amplify the signal by multiple turnovers to achieve a 13-fold improvement in sensitivity and detection limit of 50 nM. Successful signal amplification requires a temperature cycle to alternately dissociate cleaved substrate and allow fresh substrate to bind aptazyme. The results show that aptazymes have potential utility as analytical reagents for quantification of effector molecules.

Allosterically regulated ribozymes, or aptazymes, catalyze oligonucleotide cleavage reactions as the result of binding of specific effector ligands.[1] One naturally occurring aptazyme has been identified to date, the *glmS* ribozyme or catalytic riboswitch,[2] which undergoes a self-cleavage reaction in the presence of glucosamine 6-phosphate (GlcN6P).[2] The cleavage reaction is selectively activated by GlcN6P, showing no cleavage with many molecules structurally similar to GlcN6P and a substantially reduced rate of cleavage with a few analogues, such as glucosamine.[2−5] Several other aptazymes have been engineered in vitro with theophylline,[6−9] ATP,[6,10] cAMP,[11−13] cGMP,[11−13] cCMP,[13] flavin mononucleotide,[6,7,9,14] doxycycline,[15] 3-methylxanthine,[8] pefloxacin,[16] Co^{2+},[17] oligonucleotides,[18−21] and various proteins[22−24] serving as the allosteric regulator.

The coupling of a cleavage reaction with selective binding makes aptazymes appealing analytical reagents. For example, assays can be designed in which the effector ligand is detected by monitoring the cleavage reaction.[1,17,25,26] Such assays are similar to enzyme assays; but use of oligonucleotides instead of proteins for the analytical reagent could have several advantages including ease and reproducibility of production (i.e., direct chemical synthesis of the reagent) and the ability to select new targets (including synthetic compounds) in vitro using established methods. Furthermore, all such assays can be engineered to have

* To whom correspondence should be addressed. E-mail: nwalter@umich.edu; rtkenn@umich.edu.

† Current address: Department of Chemistry, Kalamazoo College, 1200 Academy St., Kalamazoo, MI 49006.

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a similar signaling molecule produced, i.e., an oligonucleotide, simplifying changing the selectivity.

Employing aptazymes in analytical assays requires the development of reliable methods for quantitatively detecting the cleavage reaction. Ideally, a detection method uses a minimal amount of sample, is rapid, can be run in parallel or array formats, and does not require elaborate design of reagents. Previous analysis of aptazyme cleavage has followed conversion of substrate into cleavage product by a variety of separations-based and real-time methods. Polyacrylamide gel electrophoresis (PAGE) has been the most widely used technique for monitoring aptazyme cleavage. Use of PAGE involves removing aliquots from a cleavage reaction at various time points, stopping the reactions, and separating them in parallel on a gel, often with detection and quantification achieved by radiolabeling the RNA. The collection of these data is slow and involves hazardous radioisotopes. Another approach involves using array formats in which aptazyme is immobilized on a surface. In this case, ligand is detected by a decrease in radioactivity as the radiolabeled portion of the aptazyme is cleaved.[17,27] While providing the potential for multiplexing, these array assays are not yet quantitative and rely on radiochemical detection. Capillary gel electrophoresis has also been used to separate and detect aptazyme cleavage in a microchip-based assay.[28] This method relies on a sieving matrix to separate cleavage products in ~10 min.

Spectroscopic methods including fluorescence,[29,30] fluorescence polarization,[31] or fluorescence resonance energy transfer (FRET) [25,26,32,33] have also been used to detect aptazyme cleavage reactions. For FRET detection, donor and acceptor fluorophores are positioned on the RNA so that they are relatively close before ribozyme cleavage, but separate after cleavage and dissociation of the 5′-cleavage product. The resulting decrease in FRET and increase in donor emission are detected to determine cleavage. Fluorescence methods provide real-time measurement and therefore are useful for monitoring molecular dynamics and the rate of cleavage reaction with good temporal resolution. These methods suffer from potential interference due to sources of fluorescence change outside of bond cleavage and the possibility that cleavage is spectroscopically silent. In the case of FRET, another disadvantage is the requirement for two fluorophores, the addition of which can be expensive and time-consuming.

Here we compare the use of FRET and rapid, free-solution CE for a small-molecule assay using the *glmS* ribozyme to detect GlcN6P as a model system. Previously, *glmS* ribozyme constructs have been used in screens for inhibitors of the GlcN6P activation using FRET[32] or fluorescence polarization[31] for detection. In our work, a minimal, trans-cleaving *glmS* construct, previously demonstrated to be similar in activity to the naturally occurring cis-ribozyme,[5] was used. (A cis-ribozyme has a substrate sequence that is part of the ribozyme strand, whereas in a trans-ribozyme the substrate is a separate strand that binds to the ribozyme.) In this system, oligonucleotide substrate is bound to the *glmS* ribozyme so that GlcN6P-activated cleavage of substrate results in three RNA molecules: the 5′- and 3′-cleavage products with three and eight nucleotides, respectively; and the unchanged ribozyme molecule, which is 23 nucleotides in length.[5] We developed GlcN6P assays using both FRET and capillary electrophoresis with laser-induced fluorescence (CE-LIF) as a readout. Methods to improve the assays by amplifying signal through a multiturnover assay are also established. Our work demonstrates the analytical utility of coupling FRET or CE with aptazymes for the detection and quantification of small molecules.

EXPERIMENTAL SECTION

Chemicals. Unless stated otherwise, all chemicals used in the experiments were purchased from Sigma-Aldrich Co. (St. Louis, MO). Tris-glycine buffer (10×) was purchased from Bio-Rad laboratories (Hercules, CA). All solutions were prepared with deionized water from an E-Pure water purification system (Barnstead International Co., Dubuque, IA). Rhodamine 110 was purchased from Molecular Probes (Eugene, OR). Substrate RNA was purchased from the Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource RNA Laboratory at the Yale University School of Medicine (New Haven, CT). Fused-silica capillary was from Polymicro Technologies (Phoenix, AZ).

Preparation of RNA. The synthetic substrate strand was deprotected as recommended by the manufacturer and purified as previously described.[34,35] For FRET measurements, the substrate strand was modified on the 5′- and 3′-ends with fluorescein (donor) and tetramethylrhodamine (acceptor), respectively, also as previously described.[36] RNA concentrations were calculated from their absorption at 260 nm and corrected for the additional absorption of fluorescein and tetramethylrhodamine by using the relations A260/A492 = 0.3 and A260/A554 = 0.49, respectively. The ribozyme strand was generated by runoff transcription from a double-stranded, PCR-amplified template that encoded an upstream T7 promoter. Transcriptions were purified as previously described,[37] and the RNA concentration was calculated as described above.

Measuring FRET and CE Cleavage Kinetics. Single-turnover cleavage reactions to compare FRET and CE for measuring kinetics were performed with samples dissolved in 50 mM HEPES-KOH, pH 7.5; 200 mM KCl; 25 mM dithiothreitol (DTT); and 20 mM $MgCl_2$. Ribozyme and substrate concentrations were 20 and 10 nM, respectively, for FRET experiments and 1000 and 500 nM, respectively, for CE experiments. The ribozyme and substrate strands were annealed at 70 °C for 2 min and then cooled to room temperature over 5 min before reacting at 25 °C. Cleavage was monitored as described below.

FRET Cleavage Assay. Steady-state FRET measurements of the *glmS* ribozyme doubly labeled with fluorescein and tetram-

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ethylrhodamine were performed on an AB2 spectrofluorometer (Thermo Fisher Scientific, Waltham, MA). Fluorescein was excited at 490 nm (4-nm bandwidth), and fluorescence emission was recorded simultaneously at the fluorescein (520 nm, 8-nm bandwidth) and tetramethylrhodamine (585 nm, 8-nm bandwidth) wavelengths, by switching the emission monochromator settings. A FRET ratio was calculated as the fluorescence of acceptor (585 nm) to donor (520 nm). Solutions (135 μL) of ribozyme and substrate at final concentrations as listed for the reactions above were annealed and transferred to a 150-μL cuvette kept at appropriate temperature by a circulating water bath. After 1 min, 15 μL of GlcN6P was added to an appropriate final concentration and mixed manually in ~5 s to initiate reaction.

Multiple-turnover cleavage experiments were prepared and monitored like the single-turnover assay above, except ribozyme and substrate concentrations were 10 and 100 nM, respectively. To cycle the temperature of sample in the cuvette, two water baths were maintained at appropriate temperatures and their connecting hoses manually switched.

CE-LIF Cleavage Assays. Single-turnover CE assays were performed under the same reaction conditions as the single-turnover FRET experiments, except ribozyme and substrate concentrations were 400 and 200 nM, respectively, and they contained 25 nM rhodamine 110 as an internal standard. Stock solutions were prepared, annealed as described above, and reactions started by adding 45 μL of stock solution to prealiquoted 5 μL of GlcN6P for appropriate final concentrations. They were incubated at 37 °C in a thermal cycler for 10 min or 30 s, as appropriate, then quenched with 10 μL of EDTA, pH 8, to a final concentration of 100 mM EDTA, and stored on ice until analysis. Multiple-turnover assays were performed under the same conditions except with 20 nM ribozyme and 200 nM substrate. They were either incubated at 50 °C or cycled between 42 and 60 °C, as described in Results and Discussion.

For kinetics analysis, a flow-gated, high-speed CE-LIF instrument similar to that described elsewhere was used.[38−40] In this system, sample in a reservoir is pumped using gas pressure through a transfer capillary to a flow gate system,[41] which allows periodic electrokinetic injection (2 kV for 1 s) onto an electrophoresis capillary where analytes were separated with 15 kV (2000 V/cm) applied. In this way, serial electropherograms are obtained at 12-s intervals to monitor reactions in the sample reservoir. Reactions were initiated by spiking 10 μL of GlcN6P sample into sample stirred by a magnetic stir bar. Sample was delivered to the flow gate at 0.8 μL/s resulting in a 7-s transfer time to the flow gate injector. An unmodified fused-silica capillary (10-μm inner diameter, 360-μm outer diameter, total length 7.5 cm, inlet to detector length 3.8 cm) was used as the separation capillary. Voltage for separation and injection was applied by a CZE 1000R high-voltage power supply (Spellman High Voltage Electronics, Plainview, NY). At the beginning of each day, the separation capillary was rinsed with 1 M NaOH, deionized water, and electrophoresis buffer for 2 min each. Electrophoresis buffer was

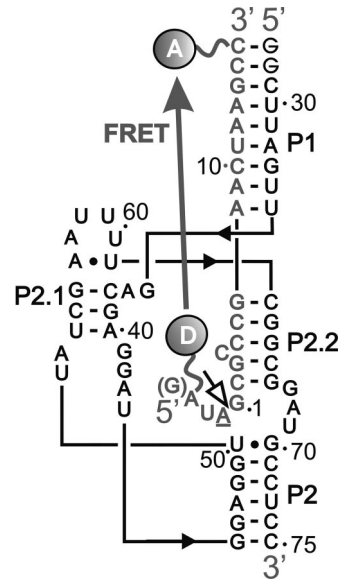

Figure 1. Structure of the minimal *glmS* ribozyme utilized here with donor (D) and acceptor (A) fluorophore attachment sites for FRET indicated. Bases in gray are substrate and bases in black are the ribozyme. The doubly labeled construct was used for FRET studies, while the substrate strand was labeled with only the 5′-fluorescein donor for CE studies. The open arrow indicates the cleavage site. The "P" designations refer to different domains as described previously.[5]

Tris−glycine−potassium buffer (25 mM tris(hydroxyamino) methane, 192 mM glycine, 5 mM K_2HPO_4).

For CE assays not requiring kinetics information, the separation capillary was 25-μm inner diameter, 360-μm outer diameter, 7-cm total length, and 3.5-cm inlet-to-detector distance. Samples were introduced onto the capillary by electrokinetic injection at 1 kV for 50 ms and separated at 12 kV in tris−glycine−potassium buffer.

Detection was accomplished using LIF as described elsewhere.[42] For excitation, the 488-nm line of an air-cooled 15-mW Ar^+ laser (Spectra Physics, Mountain View, CA) was used. Fluorescence was collected at 90° from the excitation source and spectrally filtered (520 ± 10 nm band-pass and 488-nm notch filter) and spatially filtered through two pin holes. Emission was detected by a PMT. All instrument control and data acquisition were controlled by a personal computer equipped with a multifunction board (AT-MIO-16, National Instruments, Austin, TX) using software written in-house.

Peak heights were obtained from the electropherograms using software written in-house (Cutter[43]). Normalized product peak height (product peak height divided by sum of peak heights of product and substrate) was plotted versus time to extract k_{obs} by fitting with a one-component exponential decay function (kinetics) versus the GlcN6P concentration.

RESULTS AND DISCUSSION

Single-Turnover FRET Assay. Our initial goal was to develop a procedure that allows quantitative determination of GlcN6P using FRET-detected, ligand-induced cleavage of substrate. Figure 1

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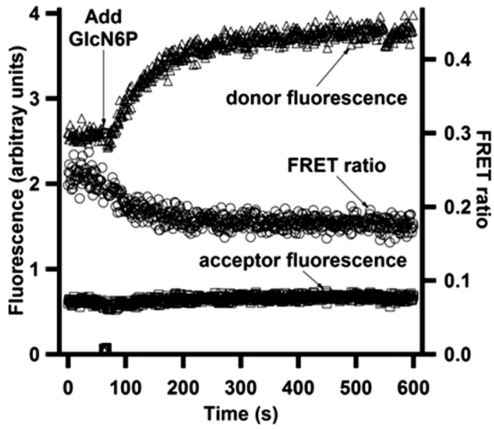

Figure 2. Detection of 100 μM GlcN6P by FRET in 50 mM HEPES−KOH, pH 7.5, 200 mM KCl, 25 mM DTT, 50 mM $MgCl_2$, with 20 nM *glmS* ribozyme and 10 nM doubly labeled substrate at 37 °C.

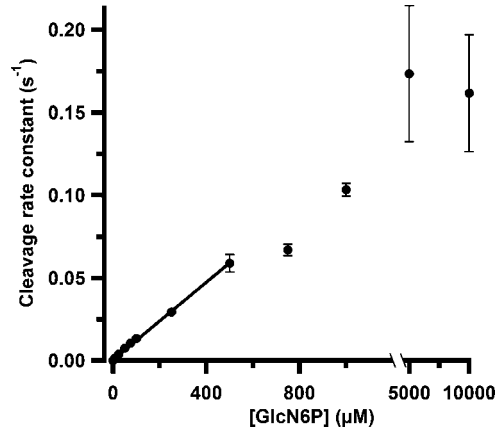

Figure 3. Calibration curve for FRET detected single-turnover cleavage by the *glmS* ribozyme. The cleavage rate constant increases linearly up to 500 μM with an R^2 value of 0.999. The limit of detection is 0.8 μM.

illustrates our minimal, trans-cleaving *glmS* ribozyme with a fluoroscein label on the 5′-end as the FRET donor and rhodamine as acceptor fluorophore on the 3′-end. This ribozyme differs from the original cis form by elimination of a loop in the P1 region (see Figure 1) so that the substrate sequence is on a separate oligonucleotide strand. In the intact ribozyme−substrate complex, the fluorophores are sufficiently close (~52 Å)[5] to allow efficient energy transfer from the excited donor to the acceptor, resulting in emission at the wavelength characteristic of the acceptor. As expected,[5] addition of GlcN6P activates the aptazyme to cleave substrate so that the 5′-product dissociates from the ribozyme−substrate complex, resulting in a fluorescein emission increase and a FRET ratio decrease (Figure 2) Acceptor signal also increases slightly, which we attribute to donor signal cross-detected in the acceptor channel. The cleavage rate was determined by fitting the FRET decrease as well as the donor fluorescence increase upon GlcN6P addition with single-exponential decay and increase curves, respectively. The measured rate constants were in good agreement; for example, at 250 μM GlcN6P, the measured rate constants were 0.032 ± 0.004 and $0.029_4 \pm 0.0006$ s^{-1} for the FRET and donor fluorescence fits, respectively. In view of their similarity, the donor increase was used to quantify the ligand-induced cleavage kinetics because of its higher signal-to-noise ratio.

Previous reports on this aptazyme used 50 mM HEPES−KOH, pH 7.5, with 200 mM KCl and 10 mM $MgCl_2$ as the reaction buffer.[32] As buffer conditions can have a significant effect on assay kinetics, we varied several parameters in an attempt to increase cleavage rates for the assay. For this study, 50 mM HEPES−KOH, 200 mM KCl, and 25 mM DTT were kept consistent together with 10 nM substrate. Other parameters were varied as follows: pH 7.5 or 8.5, $MgCl_2$ concentration from 10 to 50 mM, ribozyme concentration from 20 to 50 nM, and temperature from 25 to 60 °C. Within these limits, the best assay sensitivity was achieved with pH 7.5, 50 mM $MgCl_2$, 20 nM *glmS* ribozyme, and a reaction temperature of 37 °C. These conditions were used for further assays. With these changes, we increased the rate of cleavage from 0.095 to 0.81 min^{-1} in the presence of 100 μM GlcN6P. This rate increase is important because it can dramatically reduce the assay time.

A calibration curve for GlcN6P based on the rate constants derived from the donor-monitored cleavage activity is illustrated in Figure 3. The cleavage rate constants increase linearly with GlcN6P up to 500 μM analyte with an R^2 value of 0.999. The limit of detection (LOD), calculated as 3 times the standard deviation of the blank divided by the slope of the calibration curve, is 0.8 μM. At high concentrations of GlcN6P, above the linear dynamic range, the rapid rate of cleavage leads to high standard deviations due, at least in part, to variability in time required for analyte addition and mixing. In principle, it would be possible to add the analyte (GlcN6P) and measure the fluorescence at a fixed time instead of recording rates. However, we found that such one-time-point measurements were not reproducible. It is possible that automated addition and mixing of reagents would allow such single-time-point measurements and improve results at higher concentrations.

Single-Turnover CE Assay. As a prelude to developing a CE-LIF aptazyme assay for GlcN6P, we next evaluated the possibility that free-solution CE-LIF could be used to monitor the single-turnover reaction of our *glmS* ribozyme with a 5′-fluorescein-labeled substrate. For these experiments, the reaction mixture was continuously pumped into a flow-gated interface and analyzed at 12-s intervals by CE-LIF. The resulting electropherograms have three peaks: internal standard (rhodamine 110), ribozyme−substrate complex, and 5′-cleavage product (Figure 4A). The progress of the reaction was monitored by observing the decreasing height of the ribozyme−substrate complex peak and the increasing height of the 5′-cleavage product peak. (Peak areas would usually be preferred for quantitative analysis of the non-Gaussian peaks observed here, but the incomplete resolution of the substrate and product bands made peak height the better choice.) For quantitative analysis, these two peak heights were normalized by dividing by their sum. The reaction rate constant was obtained by plotting the normalized peak heights versus time and fitting the data with single-exponential decay and association curves (Figure 4B). The reaction rate constant in the presence of 500 μM GlcN6P was determined to be 0.0052 ± 0.0002 s^{-1}, in good agreement with the rate of 0.00603 ± 0.00008 s^{-1} determined by FRET for the same GlcN6P concentration. At 5 mM GlcN6P, the rate constant was measured to be 0.029 ± 0.001 s^{-1} by CE and 0.028 ± 0.001

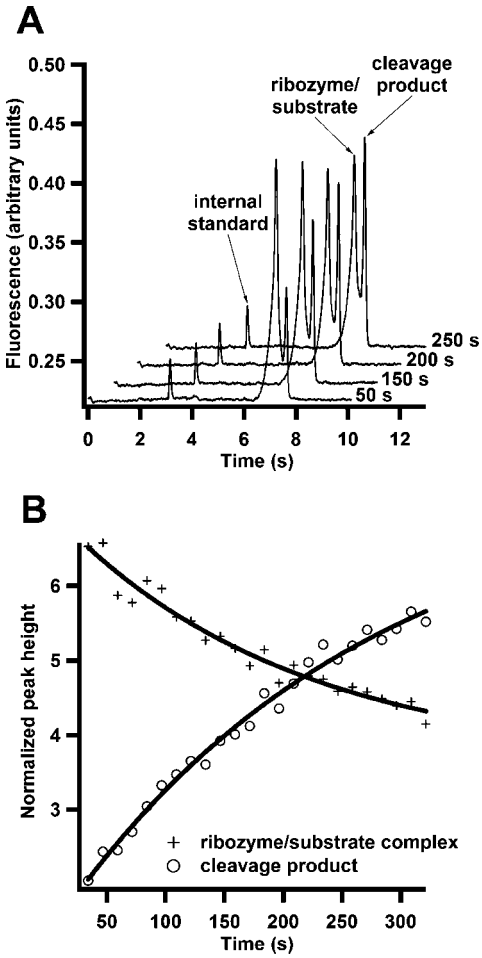

Figure 4. Detection of 500 μM GlcN6P by CE in 50 mM HEPES−KOH, pH 7.5, 200 mM KCl, 25 mM DTT, 20 mM $MgCl_2$, with 500 nM singly labeled substrate and 1 μM *glmS* ribozyme at 25 °C. (A) Electropherograms of consecutive cleavage time points, as indicated. (B) Single-exponential fits of cleavage time courses as measured by CE, yielding a rate constant ($k = 0.0052 \pm 0.0002$ s^{-1}) in good agreement with that from FRET under similar conditions (see text).

s^{-1} by FRET. These results demonstrate that rapid CE separation allows for kinetic monitoring of the ribozyme reaction.

We next evaluated the use of CE-LIF as readout for a GlcN6P assay by detecting the extent of reaction as a function of GlcN6P concentration. For the CE assay, the cleavage reaction was started and 10 min later the sample was injected into the CE for separation and detection. The separation was completed in less than 10 s, eliminating the need to quench the cleavage reaction. The use of a separation step allowed independent measurement of both substrate and product. With both of these components measured, it was possible to normalize the signal, as described above, allowing a single-time-point measurement and simplifying the assay for GlcN6P. The linear dynamic range for this calibration extends from 0.5 to 10 μM (Figure 5).

After a 10-min reaction at concentrations of GlcN6P above 10 μM, the reaction proceeded to completion. Therefore, to extend the dynamic range to GlcN6P concentrations above 10 μM, the reaction mixture was injected at 30 s instead of 10 min. At this reaction time, concentrations of GlcN6P below 10 μM are indistinguishable due to the small progression of the cleavage reaction, but concentrations greater than 10 μM are easily distinguished, resulting in a linear dynamic range from 10 to 100

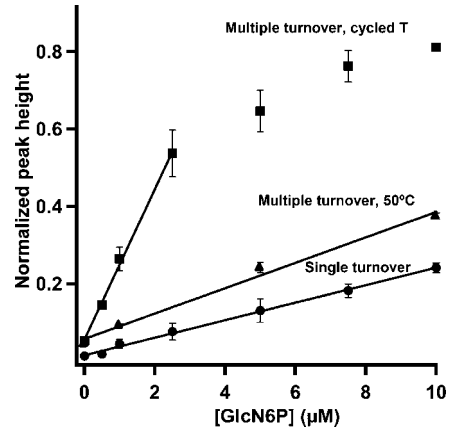

Figure 5. Calibration curves for GlcN6P using the ribozyme assay with CE-LIF for detection under different conditions as indicated on the graph. For single turnover, the assay reaction was held at 37 °C. For multiple turnover with cycled temperature, the temperature of the assay reaction was cycled between 42 and 60 °C for 10 min and 30 s, respectively, over a total time of 3.5 h.

μM. With the two calibrations, the LDR for the free-solution CE assay is 0.5−100 μM GlcN6P, with an R^2 of 0.998. The detection limit is 0.5 μM. These single-turnover assays demonstrate the potential of the *glmS* ribozyme in an assay to detect GlcN6P as well as the utility of CE in monitoring the progress of a cleavage reaction.

Both the FRET and CE methods appear to be useful for monitoring ligand-induced cleavage by the *glmS* ribozyme. For kinetic measurements, the FRET reaction achieves a 1-s temporal resolution compared to the 12 s possible by the CE-LIF method. As an assay, the FRET method has a wider dynamic range, primarily because it is based on kinetic data rather than a fixed time point like the CE-LIF method. For the same reason, the CE method was much faster, with each sample taking only seconds to analyze after the cleavage reaction, rather than minutes or hours. The CE method offers the advantage of detection of two separate peaks for the cleaved and uncleaved substrate, which allows for normalization of the signal at a single time point. This kind of normalization is not achievable by FRET. The CE-LIF approach thus offers less noise and a lower LOD and is less prone to errors due to changes in total fluorescence not associated with cleavage. The CE method uses less analyte with sample volumes as low as 10 μL compared to our 150-μL FRET samples (although the latter could be lowered with different instrumentation). The CE method uses singly labeled substrate, whereas the FRET assay requires a doubly labeled substrate.

Multiple-Turnover FRET Assay. In the assays discussed above, substrate is the limiting component of the reaction mixture so that only a single turnover of the aptazyme reaction is possible. To improve the sensitivity of GlcN6P detection by the *glmS* ribozyme, multiple-turnover reaction conditions were investigated. Under multiple-turnover conditions, excess substrate is provided, allowing the ribozyme strand to cleave multiple substrate molecules as illustrated in Figure 6A. In principle, this approach would allow generation of an amplified signal for a given effector molecule.

In our initial experiments, we used 10 nM *glmS* ribozyme, 100 nM substrate, and FRET detection to monitor the progress of the reaction. (The temporal resolution of the FRET method facilitated

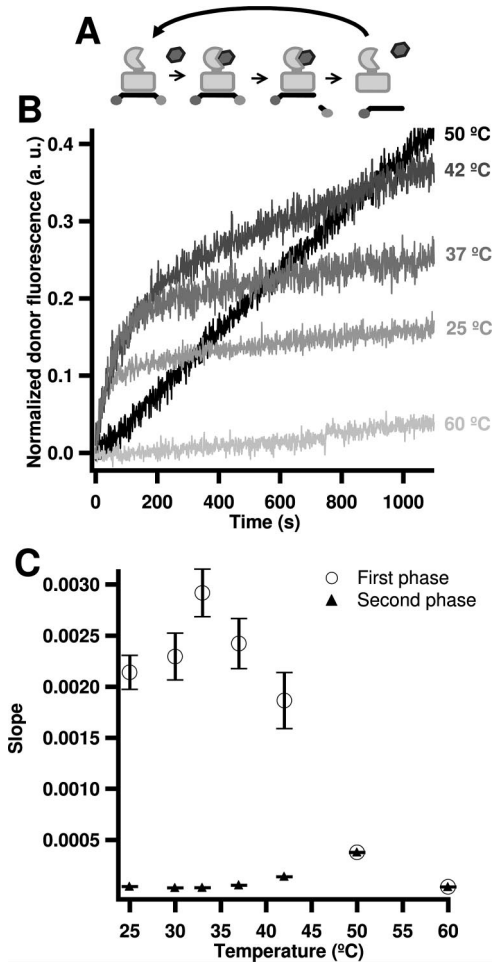

Figure 6. (A) Activity of trans-cleaving *glmS* ribozyme. (B) Temperature optimization of the multiple-turnover FRET assay. (C) Slope of linear regression fits to the first phase (first 30 points) and to the second phase (second linear range). For 50 and 60 °C, the first and second phases merge.

constant observation of the samples, which was crucial for gaining an understanding of the multiple-turnover reaction.) At 25 °C, the increase in donor signal is no greater than the increase observed under single-turnover conditions, indicating that only one substrate molecule is cleaved per ribozyme. These results suggested that the 3′-product, which binds the ribozyme with the same number of base pairs as the uncleaved substrate, did not dissociate from the ribozyme rapidly enough to allow efficient multiple-turnover events (product inhibition). We hypothesized that elevated temperatures may accelerate 3′-product dissociation and, thus, substrate turnover. Indeed, as the temperature of the cleavage reaction was increased, the extent of cleavage increased, which is evident from the increase in donor fluorescence (Figure 6B).

The temperature dependency of the fluorescence traces provides insight into the limiting steps of the multiturnover assay. At temperatures of up to 50 °C, two phases of cleavage are observed, a fast initial phase and a slow second phase. At 50 °C, the rate of cleavage becomes linear with a slope in between the former slow and fast phases. These observations suggest that the first phase reports on the first turnover where the reaction rate is limited by cleavage of prebound substrate, while the second phase reflects subsequent turnovers where slow 3′-product dissociation (or possibly fresh substrate reassociation) is rate-limiting.

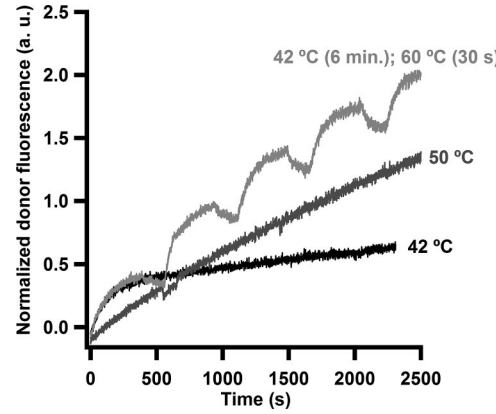

Figure 7. Multiple-turnover FRET assays with temperature cycling. Signal amplification is achieved by increasing the temperature from 42 to 50 °C. Further amplification is observed when the temperature is cycled between 42 and 60 °C as shown by the greater increase with time. Dips in the otherwise increasing donor fluorescence signal occur during the step increases to 60 °C due to a lower quantum yield of the dissociated substrate.

Examining the slope of the linear portion of each the first and second phases further supports this conclusion (Figure 6C). In the first phase, the observed rate constant increases with increasing temperature up to 37 °C and then decreases to 50 °C (at 60 °C the ribozyme is inactive). This suggests that the reaction is accelerated with increasing temperature until the rate of substrate association becomes limiting. By contrast, in the second phase, the observed rate constant continually increases to its maximum at 50 °C. This result is consistent with increasing rate of 3′-product dissociation. At this temperature, a single rate constant is observed over a broader fluorescence signal range, consistent with multiple substrate turnovers.

Our results suggested that a temperature cycling protocol could be used to achieve better signal amplification. Specifically, we developed a two-temperature program that used an elevated temperature (60 °C) to dissociate the substrate−ribozyme pair and then lowered the temperature to allow reannealing, reformation of uncleaved substrate−ribozyme−effector complex, and cleavage reaction. Figure 6 demonstrates that the fastest cleavage is achieved at 42 °C. Thus, we varied the denaturation temperatures from 50 to 60 °C (for 30 s) and the reaction time at 42 °C from 3 to 20 min while monitoring the reaction by FRET. The best conditions (i.e., with highest amplification) were found with 42 °C for 10 min followed by a 30-s pulse to 60 °C. This process of cycling between reaction and dissociation temperatures resulted in ∼4-fold signal amplification over fixed temperature at 42 °C and 2-fold at 50 °C after 40 min, as illustrated in Figure 7. This method is limited by binding of the 3′-cleavage product, which is fully complementary to the ribozyme, and thus competes with uncleaved substrate for binding as its concentration increases during the reaction (product inhibition). An alternate way to achieve multiple turnover events could involve redesign of the substrate to reduce its binding interaction with the ribozyme.

Multiple-Turnover CE Assay. The conditions for the multiple-turnover reaction that were optimized by FRET were also used in a CE assay. In this assay, ribozyme−substrate samples were either incubated at 50 °C or temperature cycled between 42 and 60 °C for 10 min and 30 s, respectively, over a total time of 3.5 h. High GlcN6P concentration samples were incubated for 5 min

either at 50 °C or temperature cycled between 42 and 60 °C for 1.5 min and 30 s, respectively. Multiple turnover at 50 °C increased the slope of the calibration curve by 2-fold compared to the single-turnover assay. With temperature cycling, a more substantial increase of 13-fold was achieved to yield a significant improvement in sensitivity (Figure 5). The LDRs for these CE-based assays are 0.5−100 μM (R^2 for 50 °C is 0.995 and 0.999 for cycled), which is the same as the range for the single-turnover assay, but the detection limit is improved to 0.1 μM for the 50 °C assay and 0.05 μM for the cycled assay. Thus, the LOD was improved ~10-fold over single-turnover conditions by using temperature cycling for the CE-LIF assay. The ultimate LOD of the assay is likely constrained by the affinity of the effector for the ribozyme, which has an apparent K_d of 200 μM,[13] as well as by the background cleavage observed in the absence of GlcN6P.

CONCLUSION

This work demonstrates the use of FRET and CE-LIF for detection of the cleavage rate of the *glmS* ribozyme in response to GlcN6P. Both FRET and CE-LIF may be used for assessing kinetics of the interaction with temporal resolution of 1 s by FRET and 12 s by CE. Both measurement methods could be used to quantify GlcN6P with a linear dynamic range from high-nanomolar and to high-micromolar concentrations and LOD of high micromolar under single-turnover conditions. Using multiple-turnover conditions, it was possible to improve sensitivity and LODs by ~10-fold in the CE-LIF assay. These proof-of-principle experiments demonstrate the potential of ribozymes for use as recognition elements in FRET and CE-based assays to quantify effector molecules. They also demonstrate the potential for enhanced sensitivity by amplification. The results support the notion that ribozymes may be useful alternatives to enzymes for a variety of assays.

Redesign of the trans-cleaving *glmS* ribozyme may improve these assays. Reducing the number of base pairs formed between the ribozyme and substrate molecules would reduce the binding energy and lower the 3′-product dissociation temperature. We hypothesize that this may achieve multiple substrate turnovers without the need for temperature cycling. Furthermore, recent work with aptazymes based on the hammerhead ribozyme demonstrates that use of the full-length ribozyme results in faster cleavage than observed with minimal structure aptazymes.[44] If these results are broadly applicable to aptazymes, use of the full-length *glmS* ribozyme may improve our assays.

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