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Pseudoknot Formation Seeds the Twister Ribozyme Cleavage Reaction Coordinate

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Abstract

The twister RNA is a recently discovered nucleolytic ribozyme that is present in both bacteria and eukarya. While its biological role remains unclear, crystal structure analyses and biochemical approaches have revealed critical features of its catalytic mechanism. Here, we set out to explore dynamic aspects of twister RNA folding along the cleavage reaction coordinate. To do so, we have employed both bulk and single-molecule fluorescence resonance energy transfer (FRET) methods to investigate a set of twister RNAs with labels strategically positioned at communicating segments. The data reveal that folding of the central pseudoknot (T1), the most crucial structural determinant to promote cleavage, exhibits reversible opening and closing dynamics at physiological Mg^{2+} concentration. Uncoupled folding, in which T1 formation precedes structuring for closing of stem P1, was confirmed using pre-steady-state three-color smFRET experiments initiated by Mg^{2+} injection. This finding suggests that the folding path of twister RNA requires proper orientation of the substrate prior to T1 closure such that the U5-A6 cleavage site becomes embraced to achieve its cleavage competent conformation. We also find that the cleaved 3'-fragment retains its compacted pseudoknot fold, despite the absence of the phylogenetically conserved stem P1, rationalizing the poor turnover efficiency of the twister ribozyme.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b01549. Synthesis of 2'-aminopropyl guanosine phosphoramidite, RNA solid-phase synthesis, HPLC time course of enzymatic ligations, EC50 curves, high FRET survival plots, representative fluorescence and FRET trajectories (PDF)

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Author Contributions

N.V. and R.B.A. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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INTRODUCTION

Nucleolytic ribozymes are catalytically active ribonucleic acids that cleave their phosphodiester backbone in a site-specific manner.^{1–4} Nine classes of nucleolytic ribozymes have been validated to date, including the recently discovered twister,^{5,34} twister sister,⁶ pistol,⁶ and hatchet⁶ motifs. Self-cleaving ribozymes have been directly linked to rolling-circle replication of RNA pathogens, processing of repetitive RNA sequences and metabolite-dependent gene regulation, while many others have unknown biological roles.⁷ Ribozyme investigations have significantly advanced our understanding of the mechanisms by which RNAs catalyze diverse chemical reactions, including the discovery of nucleobase-assisted, acid–base catalysis independent of metal ions.^{8–11}

Understanding RNA catalysis requires knowledge of the potentially intricate role that structural dynamics play in ribozyme activity.¹² Induced fit, conformational selection, active site preorganization, and substrate/product strain each invoke conformational flexibility.¹³ Experimental methods to probe thermally activated local and global structural dynamics of biomolecules are only beginning to emerge.¹⁴ Single-molecule fluorescence resonance energy transfer (smFRET) imaging has the advantage of spanning a broad range of time scales relevant to the structural dynamics of functional RNAs.¹⁵

Here, we investigate the dynamics of the *env22* twister ribozyme using smFRET imaging. High-resolution crystal structures have revealed that a central pseudoknot (T1) is formed close to the A6-U5 cleavage site that is essential for function (Figure 1).^{16–18} In contrast to T1, chemical and biochemical investigations indicate that the phylogenetically conserved stem P1 element (Figure 1) is not required for activity; twister ribozymes can efficiently cleave even a single nucleotide from the substrate 5′-end under physiological Mg^{2+} conditions.¹⁹ As these findings argue that T1 formation is a prerequisite for twister catalysis, we applied smFRET imaging to monitor the rate of twister pseudoknot formation relative to stem P1 closing using distinct structural perspectives. We show that pseudoknot formation is reversible, and that pseudoknot “breathing” contributes to the rate at which the twister ribozyme achieves cleavage–favorable conformations from which the U5-A6 sequence rapidly achieves a geometry competent for in-line attack of the U5 2′-OH at the scissile phosphate.

These findings address key questions about the folding path to the precatalytic, cleavage-competent pseudoknot as well as structural dynamics within the cleavage product that shed light on why the phylogenetically conserved stem P1 is not required for phosphodiester

cleavage and that suggest that the *env22* twister ribozyme is poorly optimized for multiple-turnover reactions.

RESULTS AND DISCUSSION

Qualitative Analysis of Pseudoknot-Dependent Cleavage Using Ensemble FRET Experiments

We first set out to probe folding and cleavage of the *env22* twister ribozyme,^{5,18} using cleavable and noncleavable bimolecular twister systems through comparative bulk FRET experiments. To measure twister folding, donor and acceptor fluorophores (LD550 and LD650, respectively) were attached at noninterfering, spatially proximal structural positions (at U2 and C31 via 2'-*O*-(3-aminopropyl) linkages; Figure 2A, Supporting Information Figure S1) based on X-ray structures.^{18,19} Similar strategies have been successfully employed for distinct RNA systems.^{20,21}

Upon addition of Mg^{2+} , the noncleavable dU5-twister construct (labeled at positions 2 and 31; tw2-31) responded by a pronounced increase in FRET (Figure 2B, left panel). This Mg^{2+} -induced change indicates twister folding into a compact structure (T1 formation), consistent with the known crystal structure (Figure 1), wherein LD550 and LD650 are expected to be in close proximity (18 Å distance between dye attachment atoms 2'-O U2 and 2'-O C31 in the folded RNA).

The rate of the observed FRET increase followed monoexponential behavior, where the estimated rate of folding, k_{fold} , was $\sim 0.29 \text{ min}^{-1}$ at 20 °C in the presence of 2 mM Mg^{2+} . This rate increased to ~ 0.42 , ~ 0.60 , and $\sim 0.84 \text{ min}^{-1}$ in the presence of 5, 10, and 20 mM Mg^{2+} , respectively. At lower Mg^{2+} concentrations, however, the terminal FRET amplitude was substantially reduced (Figure 2B, left panel, and data not shown). These data may either reflect incomplete substrate strand association and/or structural dynamics within the twister ribozyme.

Similarly, rapid increases in FRET were observed for the cleavable U5-twister upon Mg^{2+} addition (Figure 2B, right panel, Supporting Information Figure S2). However, at each Mg^{2+} concentration examined, the increase in FRET reached an attenuated maximum ($\sim 30\%$ of the noncleavable construct), followed by a modest decrease in FRET over time. In line with previously reported incomplete cleavage yields,^{18,19} each FRET signal failed to fully return to baseline. This bimodal FRET response is consistent with twister ribozyme folding and release of the LD650-containing 5 nt fragment as a consequence of phosphodiester cleavage. Given the amplitude of the FRET increase, we infer that twister folding occurs on a time scale that is similar to the cleavage reaction. These apparent rates of folding (k_{fold}) and cleavage (k_{clv}) are on the same order as those determined by ensemble 2-aminopurine fluorescence assays.^{18,19} Hence, these data imply that twister folding, and T1 formation specifically, may be rate limiting for twister ribozyme cleavage, and that strand cleavage after folding is relatively rapid.

Two-Color smFRET Setup for Twister RNA Folding Analysis

To capture twister ribozyme folding at the single-molecule scale, where substrate/ribozyme concentrations are orders of magnitude lower, we designed a *unimolecular* construct with the same labeling pattern to avoid dissociation and potential depletion of the substrate strand. To do so, we synthesized a twister construct with a shortened stem P3 that was closed by a UUCG hairpin loop (Figure 3A,B).

To generate this labeled RNA, two chemically synthesized twister half strands (19 nt and 33 nt), one tethered to LD550, the other to LD650 via the 2' hydroxyl groups of U2 and C31, respectively, were enzymatically ligated using a DNA splint and T4 DNA ligase following previously reported procedures.^{20–22} To avoid strand cleavage, the 5'-RNA half contained dU5. To provide a means for surface immobilization, the 3'-RNA strand was linked to a biotin moiety at the 3'-terminus.

Individual, surface-immobilized twister RNAs (tw2-31) were imaged using wide-field, prism-based total internal reflection microscopy, illuminating with a single-frequency light source (532 nm). Fluorescence emission from LD550 and LD650 fluorophores were simultaneously recorded using sCMOS cameras.^{23–26,32} All experiments were performed in 3-(*N*-morpholino)propanesulfonate-KOH (KMOPS), 100 mM KCl buffer at pH 7.5, in the presence of an oxygen scavenging system (Methods).

Twister RNA Folding Analyzed via T1-P1 Formation (tw2-31)

In the absence of Mg^{2+} , population histograms for tw2-31 showed a single, broad low FRET distribution (mean ~ 0.25). Increasing the Mg^{2+} concentration from 0.25, 0.5, 1.0, 2.0, to 10 mM resulted in a pronounced redistribution to a high-FRET (mean ~ 0.89) conformation, consistent with formation of the T1 pseudoknot (Figure 3B,C). This redistribution plateaued at 10 mM Mg^{2+} with approximately 70% high-FRET state occupancy. The apparent EC_{50} for this folding equilibrium was approximately 0.88 mM Mg^{2+} (Supporting Information Figure S3). As a preliminary means to quantitatively assess the kinetic and structural features of twister RNA folding, individual FRET trajectories were analyzed using a two-state hidden Markov model using Version 3.3 of the SPARTAN software package.³²

Across the Mg^{2+} titration, the absolute value of the low-FRET twister conformation increased by 0.10 (from ~ 0.25 to ~ 0.35 FRET) (Figure 3C). This finding suggests that Mg^{2+} binding to the RNA promotes a global compaction of the twister fold on a time-averaged basis. By contrast, the absolute value of the high-FRET state did not appreciably change (Figure 3C). These data reveal that the increases in FRET amplitude observed in ensemble FRET measurements (Figure 2B) reflect changes in structural dynamics within the twister ribozyme in the presence of Mg^{2+} in which the majority of twister RNAs slowly achieve an active state conformation.

At physiological Mg^{2+} concentrations (2 mM), the open and compacted conformations of the 2–31 labeled twister ribozyme are approximately equally populated (Figure 3C). Visual inspection of individual smFRET recordings revealed that molecules exhibited either predominantly low FRET or high FRET, both of which exhibited rare transient dynamics (ca. <100 ms lifetime) to higher and lower FRET states respectively (Figure 3D, left and

middle panels). These distinct classes of structural and dynamic behaviors suggest the existence of a relatively large activation barrier to twister folding. They also reveal that “breathing” dynamics of the T1 pseudoknot can occur from within the folded state to partially unfolded conformations (Figure 3B). Consistent with one or more hidden rearrangements determining the rate of complete folding and unfolding, a small fraction of molecules (~5%) were observed to reversibly exchange between low- and high-FRET states prior to the loss of FRET due to fluorophore photobleaching (average time ~ 3 s) (Figure 3D, right panel). At longer integration times (100 and 400 ms vs 10 ms) and lower laser intensity, this proportion increased to ~20 and 30%, respectively (data not shown).

Twister RNA Folding Analyzed via T1 Formation (tw31-51)

To directly image T1 formation from opposite sides of the base pairing interactions mediating the pseudoknot fold, we prepared a twister RNA labeled at the solvent-exposed 2' hydroxyl groups of nucleosides C31 and G51 (tw31-51) (Supporting Information Figure S4). Structural data predict that these anchor groups are approximately 15 Å apart in the fully folded ribozyme (Figures 1B, 4A,B). As for the tw2-31 labeled RNA, the tw31-51 ribozyme achieved high-FRET upon addition of Mg^{2+} , consistent with folding of the twister ribozyme with a Mg^{2+} EC₅₀ of ~0.61 mM (Figure 4C, Supporting Information Figure S3). Similarly, the absolute value of the lower-FRET state increased from ~0.50 to ~0.63 FRET with increasing Mg^{2+} concentrations (Figure 4C), suggesting that Mg^{2+} binding promotes a global compaction of the twister ribozyme fold. At physiological Mg^{2+} concentrations (2 mM), approximately 83% of the tw31-51 molecules adopted a high-FRET state. At higher Mg^{2+} concentrations (10 mM), high-FRET state occupancy further increased (Figure 4C), indicating nearly quantitative T1 pseudoknot formation. At 2 mM Mg^{2+} , visual inspection revealed that approximately 50% of individual tw31-51 molecules achieved long-lived (>3.5 s) high-FRET states from which transitions to lower-FRET states were rare.

Twister Ribozyme Folding Kinetics

To characterize the folding kinetics of 2–31 and 31–51 labeled twister RNAs in a manner similar to the bulk studies above (Figure 2B, left panel), we performed smFRET experiments under pre-steady state conditions at low time resolution (400 ms), recording the percentage of molecules exhibiting high FRET as a function of time after rapid injection of Mg^{2+} (5 mM) (Figure 5). In these experiments, the unimolecular tw2-31 construct achieved high-FRET at an observed rate, k_{fold} , of ~0.8 min⁻¹ (Figure 5A), consistent with the k_{fold} observed for the bimolecular system (Figure 2, ~0.4 min⁻¹).

Strikingly, the tw31-51 construct exhibited clear double-exponential growth of the high-FRET state under the same conditions: ~50% of the molecules folded rapidly ($k_{fold(1)} \sim 15$ min⁻¹), while ~50% of the molecules folded at a rate similar to the tw2-31 construct ($k_{fold(2)} \sim 0.8$ min⁻¹) (Figure 5B). The distinct kinetic signature of the tw31-51 twister ribozyme folding reaction suggests that T1 formation and structuring of the 5' substrate moiety toward T1 (likely involving P1 formation) occur on distinct time scales. We therefore hypothesized that the catalytically competent conformation of the twister ribozyme may be achieved by distinct folding pathways. In one pathway, T1 formation may occur relatively rapidly (corresponding to the faster rate), trapping the substrate strand in a nonproductive

position. In an alternative pathway, T1 formation may occur hand in hand with proper substrate positioning but at a substantially slower (ca. 15-fold) rate.

Role of Stem P1 on Pseudoknot Folding

To directly address whether stem P1 is crucial for the twister ribozyme to achieve its pseudoknot conformation, we investigated the twister 3'-cleavage product, utilizing the 31-51 fluorophore pattern (Figure 6A). This fragment provides the sequence requirements to form T1, but it cannot form P1 because it lacks the five terminal nucleosides. Strikingly, the cleaved 3'-twister product folds efficiently in the absence of stem P1, exhibiting both a lower EC_{50} of Mg^{2+} -induced folding (~ 0.26 mM; Supporting Information Figure S3) as well as a faster rate of folding compared to the full-length ribozyme (Figure 5C).

These findings support previous data showing that the twister ribozyme can cleave a single nucleotide 5' of the scissile phosphate¹⁹ and suggest that stem P1 is modestly inhibitory to the kinetics of twister ribozyme folding. They are also congruent with crystallographic analyses showing that only the nucleosides 3' of the scissile phosphate (A6 and A7) are critical for pseudoknot formation and thus substrate strand positioning.¹⁸

Distinct Structural Perspectives on the Twister Ribozyme Folding Pathway

To further define the twister ribozyme structure and dynamics during Mg^{2+} -induced folding, we investigated three additional labeling patterns. To substantiate that the tw31-51 construct specifically reports on pseudoknot formation, we investigated a twister mutant that carried a C31-U52 mismatch in T1, instead of the wild-type C31-G52 base pair (Figure 6B). In the absence of Mg^{2+} , the G52U tw31-51 construct exhibited a lower mean FRET value (~ 0.4 vs 0.5) than the wild-type tw31-51 construct (compare Figures 4C and 6B, left panels). This finding supports the notion that the FRET values exhibited by twister constructs in the absence of Mg^{2+} reflect an average of a population distribution of dynamic states. Consistent with high-FRET representing T1 pseudoknot formation, higher Mg^{2+} concentrations (>10 mM) were needed to drive folding compared to the analogous wild-type construct (compare Figures 4C and 6B, Supporting Information Figure S3). At physiological Mg^{2+} concentrations (2 mM), kinetic analysis of dynamic molecules ($\sim 32\%$ of the population) revealed an approximately 2.5-fold (Supporting Information Figure S5) decrease in the high-FRET state lifetime. These findings support the conclusion that the tw31-51 labeling pattern specifically reports on T1 formation.

In the twister crystal structures,^{17–19} stem P3 stacks coaxially on top of the continuously base-stacked T2-P2-T1-P1 segments. With the goal of defining the position of stem P3 during T1 formation, we synthesized a tw24-51 construct (Figure 6C). This RNA exhibited predominantly ($>95\%$) high FRET (mean ~ 0.75) at all Mg^{2+} concentrations tested (0–10 mM Mg^{2+}). These findings reveal that the coaxially stacked P3-T2-P2 helices observed in the crystal structure^{17–19} are largely structured even in the absence of Mg^{2+} . Hence, rearrangements of stem P3 with respect to the T2-P2 helical stack are either negligible during T1 formation (which further extends the P3-T2-P2 helical stack) or follows a trajectory that remains approximately equidistant from the active site on its path to the folded state.

In an effort to explore positioning of the U5-A6 cleavage site relative to pseudoknot T1, we synthesized a noncleavable, unimolecular tw5-51 construct, where the donor fluorophore was attached to the nonconserved and solvent-exposed dU5 residue. In the folded ribozyme, position dU5 is expected to be directly across from the T1 pseudoknot residue G51 (Figures 1 and 6D). The dU5 was labeled at the nucleobase 5-carbon position whereas G51 at its 2'-hydroxyl group, such that the dyes orient in opposite directions without structural interference in the fully folded ribozyme (Figure 1). In the absence of Mg^{2+} , the tw5-51 construct exhibited ~ 0.64 FRET, which shifted to a higher FRET state (~ 0.8) at increasing Mg^{2+} concentrations (Figure 6D). At 2 mM Mg^{2+} , approximately 10% of molecules dynamically exchanged between intermediate and high FRET states (Supporting Information Figure S6). These data are consistent with Mg^{2+} -induced compaction of an otherwise structured twister RNA. They are also in line with a folding mechanism in which the 5' substrate moiety can remain relatively dynamic prior to achieving a catalytically active fold, wherein it is embraced by the T1 pseudoknot and the U5 residue orients for an in-line attack of its 2'-OH at the scissile phosphate.^{16–19,27,28}

Folding Model for the Twister Ribozyme Derived from Three-Color smFRET

In order to definitively examine whether the twister ribozyme exhibits alternative folding pathways in which proper position of the substrate moiety is rate limiting to complete folding, we generated a unimolecular twister construct labeled for 3-color smFRET imaging, in which a LD750 dye was attached at position 2 within the tw31-51 construct (Figure 7A, and Supporting Information Figures S4D,E and S6A).

Using 532 and 640 nm alternating laser excitation (ALEX),²⁹ this constellation of FRET probes simultaneously examines two distinct degrees of structural freedom: 532 nm excitation reports on T1 formation; 640 nm excitation reports on positioning of the 5'-substrate moiety and P1 stem formation (Figure 7A,B).

In the absence of Mg^{2+} , the tw2-31-51 construct predominantly exhibited intermediate FRET values for both fluorophore pairs. Upon stopped-flow addition of Mg^{2+} (2 mM), individual FRET traces (Figure 7C) showed a rapid, modest increase in FRET consistent with compaction of the core twister fold, followed by repeated excursions to a high FRET^{31–51} state, where pseudoknot T1 appeared to form ($\sim 1–2$ s lifetime) without annealing/proper positioning of the substrate moiety (sampling). These periods of transient, reversible T1 formation persisted for tens of seconds during which time no attempts of P1 formation were observed. Following such T1 folding attempts, the majority ($>90\%$) of molecules reached the fully folded state through simultaneous T1 and P1 folding, resulting in a stably folded, high-FRET conformation for both FRET channels. In addition to this slow-folding fraction, a faster folding population was also observed in which the T1 pseudoknot and P1 stem simultaneously formed (Supporting Information Figure S7D). These two distinct folding subpopulations were quantitatively distinguished by the rates of stable T1 folding. Approximately 40% of molecules exhibited transient T1 formation (high FRET on LD550-LD650 channel) events (only T1 formed) prior to achieving a stable, fully folded conformation (both T1 and P1 formed). Analysis of the distribution of times for all T1 folding events showed that pseudoknot formation occurred at a rate (k_{sampling}) of ~ 10

min⁻¹ (Figure 7D). By contrast, analysis of P1 folding (high-FRET on LD650-LD750 channel) showed that approximately 60% of the molecules achieved a fully folded, stable high-FRET state (T1 + P1 formed), at a rate ($k_{\text{fold}}(1)$) of ~ 10 min⁻¹, while $\sim 40\%$ of the molecules folded at a nearly order of magnitude reduced rate ($k_{\text{fold}}(2)$) of ~ 2 min⁻¹ (Figure 7E). In both cases, P1 formation occurred simultaneous with T1 formation (Figure 7C, Supporting Information Figure S7). These findings provide direct evidence that the twister ribozyme can follow alternative pathways to achieve the catalytically active fold in which T1 folding can proceed, or occur concomitantly with stem P1 formation (Figure 8). By contrast, initial P1 stem formation followed by T1 pseudoknot formation, marked by a rise in LD650-LD750 FRET prior to LD550-LD650 FRET, was either forbidden or strongly disfavored.

Collectively, the present investigations reveal that T1 pseudoknot formation at the *env22* ribozyme's active site pocket is the principle determinant of proper twister folding. Moreover, our three-color smFRET measurements reveal that T1 pseudoknot can form transiently even in the absence of proper substrate strand positioning and that formation of a stable twister pseudoknot fold appears to require the substrate strand to be fully engaged within the active site. Consistent with prior evidence showing that a significant proportion of twister molecules do not cleave at elevated Mg²⁺ concentrations despite T1 formation,¹⁹ these alternative folding models predict that T1 formation in the absence of proper substrate strand positioning could be inhibitory to phosphodiester cleavage. However, at physiological Mg²⁺ concentrations, the transient nature of the T1 pseudoknot enables multiple attempts to achieve the catalytically competent fold.

Our findings further reveal that the cleaved product can maintain its compacted pseudoknot fold, suggesting that the twister ribozyme may not be evolutionarily optimized for multiturnover reactions.

In summary, our study defines structural and kinetic features of the functional twister ribozyme core that shed important new light on Mg²⁺-dependent pseudoknot formation and structural dynamics within this small RNA that are likely to strongly influence its catalytic activity. The precise role and relevance of these alternative folding pathways, and the physical nature of the folding intermediates observed, as they pertain to the biological activities of the twister ribozyme, warrant further investigation.

MATERIALS AND METHODS

Solid-Phase Synthesis of Prefunctionalized RNA

RNA Solid-phase synthesis^{30,31} of 2'-*O*-aminoalkyl-, 3'-*O*-biotinylated-, 5'-*O*-phosphorylated-, and/or 5-aminoallyl-modified oligoribonucleotides were performed as described in the Supporting Information Methods.

Preparation of Fluorophore-Labeled RNA

LD550 and LD650 NHS ester were obtained from Lumidyne Technologies. DMSO was dried over activated molecular sieves. Dye-NHS ester (100 nmol) was dissolved in anhydrous DMSO (2 μ L). Lyophilized RNA (40 nmol) containing a 2'-*O*-(3-aminopropyl) nucleoside modification was dissolved in water (30 μ L) and desalted by precipitation using

sodium acetate buffer ($\sim 6 \mu\text{L}$; 1 M, pH 5.3) and absolute ethanol ($\sim 90 \mu\text{L}$). The suspension was kept for 2 h at -80°C , followed by centrifugation for 30 min at 4°C at 13,000 RPM (Eppendorf 5430R, rotor F-45-30-11). The supernatant was pipetted off, the pellet was resuspended in absolute ethanol ($\sim 90 \mu\text{L}$) and centrifugation was repeated. Ethanol was again pipetted off and the pellet was dried on the high vacuum. Subsequently, RNA was dissolved in labeling buffer ($40 \mu\text{L}$; 100 mM sodium-borate buffer, pH 8.5), with a final concentration of c_{RNA} of 1 mM. The corresponding volume of the dye-NHS ester solution ($2 \mu\text{L}$) was added to the RNA solution (to reach a concentration of $c_{\text{Dye}} = 2.38 \text{ mM}$ in the final reaction volume). The reaction mixture was kept at room temperature in the dark for 2–3 h. The reaction was stopped by precipitation with sodium acetate buffer ($\sim 8 \mu\text{L}$; 1 M, pH 5.3) and absolute ethanol ($\sim 120 \mu\text{L}$) for 2 h at -80°C followed by centrifugation for 30 min at 4°C at 13 000. The colored pellets were dried, resuspended in water, and purified by anion-exchange chromatography on a Dionex DNAPac100 column ($4 \times 250 \text{ mm}$) at 60°C . Flow rate was 1 mL/min; Buffer A: Tris-HCl (25 mM), urea (6 M), pH 8.0. Buffer B: Tris-HCl (25 mM), urea (6 M), NaClO_4 (0.5 M), pH 8.0; gradient was 0–60% B in A within 45 min; UV detection was at a wavelength λ of 260 nm (RNA), 550 nm (LD550), 650 (or 595) nm (LD650) and 750 nm (LD750). Fractions containing labeled oligonucleotide were loaded on a C18 SepPak cartridge (Waters/Millipore), washed with 0.1 M $(\text{Et}_3\text{NH})^+\text{HCO}_3^-$ and H_2O , eluted with $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (1/1), and lyophilized to dryness.

Enzymatic Ligation

Twister RNA containing 5'-biotinylated, and LD550/LD650 labels were prepared by splinted enzymatic ligation of two chemically synthesized fragments (Supporting Information Figure S4) using T4 DNA ligase (Fermentas).²² Briefly, $10 \mu\text{M}$ of each RNA fragment (8 nmol), $10 \mu\text{M}$ of a DNA splint oligonucleotide (IDT, 8 nmol), and water ($530 \mu\text{L}$) were heated at 90°C for 2 min and passively cooled to room temperature before adding $10\times$ ligation buffer ($80 \mu\text{L}$, Fermentas) and PEG ($80 \mu\text{L}$, Fermentas). T4 DNA ligase ($80 \mu\text{L}$; Fermentas, $5 \text{ U}/\mu\text{L}$) of a final concentration of $0.5 \text{ U}/\mu\text{L}$ in a total volume of 0.8 mL was incubated for 5 h at room temperature or 37°C . Ligation was stopped by phenol/chloroform extraction. Analysis of the ligation reaction and purification of the ligation products were performed by anion exchange chromatography and the final product confirmed by LC-ESI mass spectrometry.

smFRET Experiments

smFRET data were acquired using a prism-based total internal reflection microscope, where the biotinylated twister ribozyme was surface immobilized within PEG-passivated, streptavidin-coated quartz microfluidic devices.²³ The LD550 fluorophore was directly illuminated under $\sim 20\text{--}800 \text{ W cm}^{-2}$ intensity at 532 nm (Laser Quantum). In three-color ALEX experiments, LD550 and LD650 were illuminated with similar intensity at 532 and 640 nm (Coherent), respectively, utilizing shutters to alternate excitation modes (Uniblitz). Photons emitted from LD550, LD650, and LD750 were collected using a 1.27 NA $60\times$ Plan-APO water-immersion objective (Nikon), where optical treatments were used to spatially separate LD550, LD650, and LD750 frequencies onto synchronized sCMOS devices (Flash 4.0, V2; Hamamatsu). Fluorescence data were acquired using home-built acquisition software coded in LabVIEW (National Instruments) at a rate of 100 frames per second (10

ms integration), unless otherwise specified. Fluorescence trajectories were selected from the movie files for analysis using SPARTAN image analysis software³² coded in MATLAB (The MathWorks). Fluorescence trajectories were selected on the basis of the following criteria: a single catastrophic photobleaching event, at least 6:1 signal-to-background noise ratio calculated from the total fluorescence intensity, and a FRET lifetime of at least 50 frames (500 ms) in any FRET state > 0.15 . Two-color smFRET trajectories were calculated from the acquired fluorescence data using the formula $\text{FRET} = I_{\text{LD650}} / (I_{\text{LD550}} + I_{\text{LD650}})$, where I_{LD550} and I_{LD650} represent the LD550 and LD650 fluorescence intensities, respectively. For three-color ALEX experiments, smFRET trajectories were calculated as previously described.²⁹ All smFRET experiments were performed in 50 mM 3-(*N*-morpholino) propanesulfonate-KOH (KMOPS), 100 mM KCl, pH 7.5, 1 mM BME buffer in the presence of 1 unit of protocatchuate-3,4-dioxygenase and 2 mM protocatchuic acid,²⁴ in the absence of solution triplet state quenchers at 25 °C. Concentrations of MgCl₂ and twister were as specified in the individual figure captions. FRET state occupancies and transition rates were estimated by idealization to a two-state Markov model using the segmental k-means algorithm.^{32,33}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Structure of the *env22* twister ribozyme (pdb 4RGE). (A) Side view: Substrate RNA (green) and ribozyme strand (gray) indicating the U5-A6 cleavage site (yellow), stem-loop P4 (red), and pseudoknot T1 (blue) regions. Nucleotides 2, 5, 24, 31, and 51 are highlighted as they served as the fluorophore anchors for FRET spectroscopy. (B) Front view and distance matrix (Å) of nucleoside atoms to which fluorophores were attached (2'-O of U2, U24, C31, and G51 via aminopropyl tethers, and carbon-5 of dU5 via an aminoallyl tether). (C) Back view (left) and cartoon presentation (right). Black triangles indicate the scissile phosphate.

Figure 2.

Tracking substrate annealing, folding, and cleavage of a bimolecular twister ribozyme using ensemble FRET spectroscopy. (A) *env22* twister sequence and secondary structure; pseudoknots T1 and T2 (blue), stems P1–P4, cleavage site U5–A6 (yellow), indicating fluorophore labeling positions: LD550 (green) and LD650 (red). (B) FRET time course of the noncleavable dU5 twister ribozyme (left) following stopped-flow Mg^{2+} addition (2, 5, or 10 mM final concentration); FRET time course of cleavable U5 twister (right) following stopped-flow Mg^{2+} addition (2, 5, or 10 mM final concentration). Conditions: $\alpha(\text{RNA})_{\text{each strand}} = 0.5 \mu\text{M}$, 50 mM KMOPS, pH 7.5, 20 °C.

Figure 3. Noncleavable, unimolecular twister with the U2-C31 labeling pattern as in Figure 2. (A) Sequence and secondary structure. (B) Schematics of labeling pattern and anticipated dynamics. (C) Population FRET histograms showing the mean FRET values and FRET state occupancies (%) observed at increasing concentrations of Mg^{2+} (as indicated) at 10 ms time resolution. (D) Representative fluorescence (LD550, green; LD650, red) and FRET (blue) trajectories at 2 mM Mg^{2+} , idealized to a two-state Markov model (red).

Figure 4. Noncleavable twister with U31-G51 labeling pattern. (A) Sequence and secondary structure. (B) Schematics of labeling pattern and anticipated dynamics. (C) Population FRET histograms showing the mean FRET values and FRET state occupancies (%) observed at varying concentrations of Mg^{2+} (as indicated).

Figure 5.

Comparison of folding kinetics between U2-C31 and C31-G51 labeled twister determined by smFRET spectroscopy. (A) The rate of folded state occupancy (high-FRET) following stopped-flow Mg^{2+} (5 mM) addition fit to a single-exponential process (black line) (B) Same as (A), but for *tw31-51* fit to a double-exponential process (black line). (C) Same as (B), but for *tw31-51 ΔP1* (3'-cleavage product). Data points overlaid for three independent measurements.

Figure 6. Noncleavable twister ribozyme RNA controls (A) tw31-51 Δ P1 (3'-cleavage product). (B) tw31-51 G52U mutant. (C) tw24-51. (D) tw5-51. Schematics of anticipated dynamics (upper panels); contour plots and population FRET histograms showing the mean FRET values and FRET state occupancies (%) observed at selected concentrations of Mg^{2+} (as indicated).

Figure 7.

Three-color smFRET imaging of the tw2-31-51 twister ribozyme. (A) Labeling pattern and cartoon of anticipated dynamics. (B) Schematics of alternating laser excitation (ALEX) experiments. (C) Representative FRET time course after stopped-flow Mg^{2+} addition (2 mM final concentration: dotted line in c; arrows in d and e). (D) Distribution of times to the first T1 sampling event. (E) Distribution of times to achieve a catalytically active fold.

Figure 8. Alternative folding pathways for the twister ribozyme to achieve its catalytically active, pseudoknot fold (precleavage structural dynamics; yellow background). The cleaved 3' twister fragment retains its pseudoknot fold in the absence of stem P1 (postcleavage dynamics; gray background).