Single-molecule studies of group II intron ribozymes

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Group II intron ribozymes fold into their native structure by a unique stepwise process that involves an initial slow compaction followed by fast formation of the native state in a Mg2+-dependent manner. Single-molecule fluorescence reveals three distinct on-pathway conformations in dynamic equilibrium connected by relatively small activation barriers. From a most stable near-native state, the unobserved catalytically active conformer is reached. This most compact conformer occurs only transiently above 20 mM Mg2+ and is stabilized by substrate binding, which together explain the slow cleavage of the ribozyme. Structural dynamics increase with increasing Mg2+ concentrations, enabling the enzyme to reach its active state.

RNA molecules exhibit very diverse and fascinating folding pathways from random coil to native state. Smaller RNAs fold rapidly and smoothly after an apparent two-state process (1), whereas large RNAs sample intermediate conformations before reaching the active structure (2). Generally, a fast (1–50 ms) overall compaction into a stable but misfolded kinetic trap is found in an early stage of the folding pathway (1, 3–7) followed by slower rate-limiting folding to the native state (2).

Group II introns rank among the largest ribozymes in nature and display remarkable features distinct from other large RNAs (8, 9). These ribozymes are self-splicing multidomain RNA molecules exhibiting a rich evolutionary heritage, the parallels in structure and catalytic mechanism with spliceosomal RNA being only one example (9–11). Their overall secondary structure organization into six modular domains is generally preserved (Fig. 1†) (8). Only nucleotides involved in tertiary contacts are conserved, but these are dispersed throughout the secondary structure, unlike in other large RNAs (12, 13). Another specific property of group II intron ribozymes is the overall high Mg2+ requirement for maximal activity in vitro (14, 15).

The D135 ribozyme is derived from the Saccharomyces cerevisiae group II intron Sc:ai5y and contains the necessary components for catalysis (9), allowing efficient and selective cleavage of RNA substrates with multiple turnover (14). This ribozyme has been used as a model system to study folding and metal ion binding of large RNAs (14, 16, 17). Until now, the folding pathway of D135 has been described as an apparent two-step process involving one obligatory intermediate (U ↔ I ↔ N) under optimal conditions for splicing (42°C, 500 mM KCl, 100 mM MgCl2) (17–19). The unfolded state U is an extended but structurally defined conformer of D135 in the presence of monovalent ions and possesses extensive secondary structure but no tertiary interactions (17, 19). The first folding step (kobs ≈ 1 min−1) consists of a critical rate-limiting compaction within domain I (D1, Fig. 1) to a folding intermediate I (17, 19, 20). The second step of domain assembly is not well characterized, but it is generally believed that the remaining domains fold rapidly into their native position onto the D1 scaffold (18).

Unlike other large RNAs, the group II intron folding is unique because the initial tertiary collapse of D135 is slow and I is not a kinetic trap but an obligatory folding intermediate (19). Structural and dynamic information about this intermediate is lacking. To elucidate the structural dynamics of the second folding step by single-molecule Förster resonance energy transfer (FRET), we have designed a fluorophore-labeled D135 construct (D135–L14) by introducing two modular loops (21) into D1 and D4 that specifically bind two DNA-oligonucleotides carrying the FRET pair Cy3 (Cy3-DNA) and Cy5 (Cy5-DNA), respectively [Fig. 1A and supporting information (SI) Figs. S1 and S2†]. Our single-molecule experiments reveal three distinct conformations of D135–L14 in dynamic equilibrium under a wide range of Mg2+ concentrations and a previously undetected on-pathway folding intermediate. The native conformation is scarce and becomes apparent only at 20 mM Mg2+ or higher. However, this high-FRET state is stabilized by substrate binding, indicating that this is actually the catalytically active conformation in vitro. The folding rates between these conformations are fast and exhibit only a subtle Mg2+ dependence, in agreement with a recent ensemble averaged study (22), indicating that these conformations are connected by small activation barriers with similar, but not identical, Mg2+ affinities. This is the largest protein-free ribozyme studied by single-molecule fluorescence so far and exemplifies how single-molecule approaches can be applied to characterize the folding pathway of very large ribozymes in detail. Interestingly, the ribozyme dynamics increase with Mg2+ concentration, making the active state accessible.

Results

Fluorophore-Labeled D135–L14 Ribozyme Is Catalytically Competent and Requires Mg2+ for Folding. We tested the activity of the in vitro transcribed (23) D135–L14 construct in a single-turnover cleavage assay with the substrate 17/7 under optimal conditions for self-splicing (17) (Fig. S3A and SI Results). The labeling scheme only minimally affects the cleavage activity of the ribozyme. D135–L14 yields a cleavage rate constant kobs = 0.37 ± 0.02 min−1, which is reduced in the presence of the fluorophore- and biotin-labeled DNA-oligonucleotides by only 27%. The final amount of product formation was 85% for all constructs. kobs depends on [Mg2+] giving a dissociation constant Kmγ = 52.7 ± 3.4 mM, comparable with reported values (9, 14).

Folding in bulk was also measured by FRET (Fig. 2). Upon addition of Mg2+ ions to fluorophore labeled D135–L14, the donor intensity decreases while the acceptor increases (Fig. 2A). The overall FRET ratio increases exponentially with kobs = 0.90 ± 0.07 min−1 (100 mM MgCl2, Fig. 2B), in agreement with reported values (14, 18, 19). The FRET increase suggests that a conformational change takes place in the presence of Mg2+, during which the relative distance between the fluorophores on the RNA decreases. The observed FRET increase is Mg2+-dependent, giving again a Kmγ = 42.3 ± 1.3 mM (Fig. 2C).

Finally, we confirmed efficient and specific binding between the

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DNA-oligonucleotides and D135–L14 by nondenaturing gel electrophoresis (Fig. S2).

Taken together, the above results demonstrate that the fluorophore-labeled D135–L14 behaves like the D135 ribozyme regarding catalysis, folding, and Mg2⁺ requirement.

**Single-Molecule FRET Reveals (smFRET) Three Conformations in Equilibrium.** Single-molecule FRET offers the potential to monitor and identify transient folding intermediates without the need for synchronization and can help elucidate the folding pathways of ribozymes. Here, we have used an approach similar to our earlier studies with the hairpin ribozyme (24, 25) to study the folding dynamics of the much larger D135–L14 ribozyme.

Fluorophore-labeled D135–L14 ribozymes were incubated in the presence of specific amounts of Mg2⁺ at 42°C to induce folding before immobilization on the quartz surface via a biotinylated DNA-oligonucleotide hybridized to the 3’ end tail (Figs. 1A and 3A and Fig. S1). Characteristic time trajectories of such surface immobilized D135–L14 ribozymes in 10 mM and 100 mM Mg2⁺ at room temperature are shown in Fig. 3 B and C. The observed FRET ratios jump stochastically between three values $\sim$0.25, $\sim$0.4, and $\sim$0.6. Hence, the D135–L14 ribozyme adopts at least three distinguishable structural conformations [extended intermediate (I), folded intermediate (F), and native (N)] in dynamic equilibrium. We assigned the low-FRET state to I and not U (Fig. 1), folded intermediate (F), and native (N) in dynamic equilibrium, as evidenced by the FRET time trajectories and the dwell time spent in each state. At 10 mM Mg2⁺ and below, dynamic molecules stay predominantly in the I state, whereas at 100 mM Mg2⁺ the F state prevails (Fig. 3 B and C). To quantify the effect of Mg2⁺ on the stability of each state, we calculated FRET distributions from $\sim$150 single-molecule trajectories at Mg2⁺ concentrations ranging from 1 to 100 mM (Fig. 4). At 1 mM Mg2⁺ the D135–L14 ribozyme resides exclusively in the I state, and no structural dynamics are observed. We can easily distinguish this state from complexes lacking the acceptor fluorophore (caused by photobleaching or blinking) because the observed FRET ratio for the latter molecules is $\sim$0.1 (Fig. 4, Top). The distributions for the F and N states become apparent only above 10 and 20 mM Mg2⁺, respectively (Figs. 3 B and C and 4). The N state is never populated for extended times, which is reflected as a low-amplitude distribution in the histograms. The amplitude of the F state distribution...
Fig. 3. smFRET analysis of D135–L14 ribozymes. (A) Principle of total internal reflection fluorescence spectroscopy (TIRF). Molecules are immobilized on a quartz slide and placed in the microscope. The fluorophores are excited by the laser's evanescent wave above the critical angle, giving emission signals collected through the objective with a high-quantum yield camera. (B) smFRET time trace and histogram at 10 mM MgCl2 showing two conformational states at 0.25 and 0.4. (C) smFRET time trace and histogram at 100 mM MgCl2 sampling the three FRET states 0.25, 0.4, and 0.6. (D) (Left) smFRET time trace shows a dynamic behavior only upon increase in MgCl2 concentration from 10 to 100 mM at 50–58 s. (Right) Distributions of the states before and after the addition of MgCl2.

Fig. 4. Histograms of FRET efficiency values from single-molecules traces in dependence of MgCl2 concentration. All traces (static and dynamic) were used to build the histogram at 1 mM, whereas all other histograms were created from traces showing dynamics only. Three distributions centered at FRET ~0.25, ~0.4, and ~0.6. The peaks of the 0.25 and 0.4 states show a slight shift to lower FRET with higher MgCl2 concentration. The 0.6 distribution arises above 15–20 mM and is only minimally but steadily populated. Gaussian fits for the individual and the sum of all distributions are shown as solid lines. The dashed bars at 0.12 FRET correspond to the distribution in absence of acceptor (Top).

Peaks of the FRET Histogram Distributions Decrease Slightly with MgCl2. Close inspection of the histograms (Fig. 4) reveals that the peak distributions of the I and F states shift to slightly lower FRET values with increasing MgCl2. The I state distribution shifts from ~0.27 (1 mM Mg2+) to 0.24 (20 mM Mg2+), whereas the F state distribution shifts from 0.45 (10 Mg2+) to 0.40 (20 mM Mg2+). At lower Mg2+ concentrations, the F and N states are possibly in fast dynamic equilibrium (lifetime <30 ms), and as a result we observe only a weighted-average FRET ratio between F and N. Alternatively, the I and F states each become structurally less compact above 1 mM and 20 mM Mg2+, respectively, giving rise to a shorter distance between the two fluorophores. Neither of the two hypotheses can be ruled out at the moment, but this result supports the idea that the group II intron conformations are connected by relatively small activation barriers.

increases from 0.04 to 0.08 with increasing [Mg2+] whereas the amplitude of the low FRET decreases from 0.23 to 0.06. This indicates that the relative stability of the F state increases concomitantly with the decrease of the I state. This antiparallel behavior, however, is not very dramatic because both states are still present between 10 and 100 mM Mg2+. The N state distribution amplitude remains constant above ~50 mM Mg2+ (see below).

To learn more about the Mg2+ requirement for these structural dynamics, we monitored the folding of a single D135–L14 ribozyme while changing the buffer conditions from 10 to 100 mM MgCl2 (Fig. 3D). During the first 50 s, the ribozyme kept in 10 mM MgCl2 displays a FRET ratio ~0.25 (I state) matching the FRET histogram obtained under steady-state conditions (Fig. 4, Top). Between 50 and 58 s we exchanged the buffer in the microscope slide by using a syringe pump to increase the Mg2+ concentration to 100 mM. After a dead time of ~10 s, the FRET ratio jumps to 0.4, indicating that a Mg2+-induced conformational change to the F state takes place. The structural dynamics of the ribozyme increase as evidenced by FRET jumps between the 0.25 and 0.4 FRET states. The FRET histogram corresponding to the high-[Mg2+] period also matches the FRET histogram obtained in steady-state conditions (Fig. 4, Bottom). This experiment clearly shows that the individual FRET states are directly dependent on the Mg2+ concentration and that the D135–L14 ribozyme readily reacts to an increased Mg2+ background by reaching a higher FRET state.

The presence of both the F and N states only above 20 mM Mg2+ coincides with the onset of catalytic activity (compare Fig. 4 and Fig. S3), suggesting that one of these two conformations corresponds to the catalytically active structure. It has been suggested that D135 collapses directly to the active state with a midpoint of ~20–40 mM Mg2+ (14).
Substrate Binding Stabilizes the 0.6-FRET Conformation. To determine which state (F or N) represents the active conformation, we performed smFRET studies in the presence of the substrates 17/7 and 17/7-dC (2′-deoxy at cleavage site) (26). The slow cleavage of 17/7-dC \(k_{obs} = 0.05 \pm 0.01 \text{ min}^{-1}\) enabled us to observe the substrate-bound D135–L14 molecules for longer periods of time. The presence of either 17/7 or 17/7-dC clearly stabilizes N (Fig. 5), suggesting that the high-FRET state corresponds to the active conformation. Consequently, F represents a hitherto undetected obligatory intermediate, or near-native state, from which only few molecules reach the active state at a time. The formation of the e-e′ and λ-λ′ tertiary contacts and the two intron and exon binding sequences (IBS-EBBS) interactions between the substrate and D1 appear to be essential to stabilize the active conformation. The wild-type (WT) substrate 17/7 leads to a higher occurrence of the 0.6 state compared with the 17/7-dC oligonucleotide (Fig. 5A). A plausible explanation for this observation could be that the 2′-OH at the cleavage site makes contacts that contribute to assembly of the catalytic core and hence also to the overall architecture of the ribozyme.

The assignment of the 0.25 state to I (D1-folded), the 0.4 state to F (compact D135), and the 0.6 state to the native structure is further supported by a recent study (22). Under low-salt conditions, D1 folds to the near-native state to which D3 and D5 can dock. However, docking is not stable, and the folding pathway arrests primarily at the I state. This lack of structural stability can be overcome by increasing the Mg\(^{2+}\) concentration \textit{in vitro} or the participation of proteins \textit{in vivo} (9). The occurrence of the 0.4 state only at higher [Mg\(^{2+}\)] clearly assigns the I and F state to folded DI and compact D135, respectively.

\textbf{Mg\(^{2+}\) Ions Increase Structural Dynamics to Form the Active State.} It is generally assumed that the addition of Mg\(^{2+}\) to RNA initiates folding and leads to a compact and more rigid structure. Here, we detect the opposite and so far unobserved behavior. Although Mg\(^{2+}\) clearly initiates folding of D135 leading to the more compact I, F, and N states, the structural dynamics increase considerably with higher [Mg\(^{2+}\)]. At very low [Mg\(^{2+}\)] the molecules remain static in the I state, but at higher concentrations an increasing number of transitions among the three states is observed. The static molecules depict constant FRET values of \(\approx 0.25\) (i.e., the I state) at all Mg\(^{2+}\) concentrations >1 mM tested (compare Fig. 4 and Fig. S5). It is possible that these static molecules correspond to a subpopulation of molecules that display a very slow \(k_1 \approx 0.005 \text{ s}^{-1}\), slower than our \(\approx 200\text{-s observation window, but we cannot distinguish between these two possibilities. The fraction of dynamic molecules versus [Mg\(^{2+}\)] (Fig. 6A) yields a dissociation constant \(K_{D_{Mg}} = 41.4 \pm 1.4 \text{ mM}\), which coincides with the values for bulk folding and cleavage (Fig. 2C and SI Results). Even at high [Mg\(^{2+}\)], the fraction of dynamic molecules does not exceed 50% in apparent discrepancy with the cleavage experiments of the same D135–L14 construct (see above). However, the cleavage assays are conducted at 42°C, whereas the smFRET experiments are done at 22°C for technical reasons. This finding suggests that the higher temperature is needed to enable the remaining molecules reach the active conformation. Taken together, these two results clearly link the structural dynamics of D135 to its function, raising the interesting possibility that domain motion contributes to successful reactivity.

\textbf{Dwell-Time Analysis Determines the Rates of Folding.} To quantify the relative stability of each conformation, we determined the rates of folding \(k_1, k_{-1}, k_2, \) and \(k_{-2}\) for the ribozyme. The dwell time in each state between two conformational transitions from \(\approx 150\) single-molecule time trajectories was measured, and dwell time distributions for each state were calculated. Static molecules in the I state could not be included in this analysis. Dwell times in the 0.4 FRET state were divided into two distributions depending on the final state (I or N) to determine \(k_{-1}\) and \(k_2\), respectively. Fig. 6B shows a characteristic dwell time \((\tau)\) distribution for the I state in 50 mM Mg\(^{2+}\). The distribution decays exponentially as expected for a stochastic process. To obtain \(k_1\), we integrated the distribution into a cumulative time distribution (Fig. 6C), which was then fit to an exponential growth curve, as described for the two model solutions (24, 25, 27). The integrated distribution reveals folding heterogeneity in the form of two distinguishable exponential, as observed for other ribozymes (25, 28–30). The major component exhibits an exponential rate \(k_1 = 0.66 \text{ s}^{-1}\). The folding rate \(k_{-1} = 0.51 \text{ s}^{-1}\) obtained similarly also exhibited heterogeneity. The measured rates in 100 mM Mg\(^{2+}\) are in agreement with the FRET histogram distributions (Fig. 4).
Bottom). The ratio $k_1/k_{-1} = 1.3$ corresponds with the ratio of distribution amplitudes for the F and I states, $A_{0,1}/A_{0,2} = 0.78/0.56 = 1.4$. Please note that in an ensemble experiment the observed folding rates would be slower than those reported here because of averaging between the static and dynamic subpopulations. This illustrates the strength of single-molecule experiments, which enable us to uncover heterogeneity in folding and thus elucidate details of the mechanism otherwise hidden by ensemble averaging.

Lacking a statistically significant number of I to N transitions because $k_2 < k_{-1}$ and the high FRET conformation is only rarely reached, $k_2$ and $k_{-2}$ could not be determined with dwell time distributions. To estimate their values, we measured the average dwell time in I before jumping to N, and in N before jumping back to I for 19 molecules (tav = 15.3 and 0.6 s, respectively). We calculated $k_2 = 0.07$ s$^{-1}$ and $k_{-2} = 1.7$ s$^{-1}$ as the inverse of these values (Fig. 1B). The ratio $k_2/k_{-2} = 0.04$ is in reasonable agreement with the ratio of relative amplitudes of the 0.6 and 0.4 FRET distributions ($A_{0,2}/A_{0,4} = 0.008/0.078 = 0.10$, Fig. 4), confirming the low abundance of N in absence of the substrate. We also measured $k_2$ and $k_{-2}$ in presence of 17/7 and 17/7-dC from 6 and 17 single-molecule trajectories, respectively. Substrate binding increases $k_2$ by nearly 10-fold (0.8 and 0.7 s$^{-1}$, respectively) and decreases $k_{-2}$ by $\sim 50\%$ (1.1 and 1.2 s$^{-1}$, respectively), further indicating that the substrate stabilizes folding into the N state.

**Forward Rates Are Independent of Mg$^{2+}$.** We determined the folding rates in the absence of substrate between 5 and 100 mM [Mg$^{2+}$] (Fig. 6D). Below this concentration, the ribozyme is mostly static in the I state, and thus, not enough dynamic events were observed to quantify the rates with confidence. We found evidence of folding heterogeneity for $k_1$ and $k_{-1}$ at all concentrations (Fig. S6 and Tables S1 and S2). At 5 mM Mg$^{2+}$, the major component of $k_{-1}$ is 1.5-fold larger than the major component of $k_1$, consistent with I being more stable than F at low Mg$^{2+}$ (Fig. 6D). $k_1$ remains approximately constant, whereas $k_{-1}$ first increases and then decreases at [Mg$^{2+}$] $>40$ mM, shifting the equilibrium from I toward F (Tables S1 and S2). This finding is consistent with the change in relative stability observed in the FRET histogram distributions (Fig. 4). The rate constants $k_2$ and $k_{-2}$ were also found to be approximately constant over the Mg range tested.

**Discussion**

**Folding of Large Multidomain RNAs.** Here, we have used the group II intron ribozyme as a model system to study folding of large multidomain ribozymes. Our single-molecule experiments reveal important concepts for RNA folding in general resolve long-standing questions for group II intron catalysis.

The group II intron ribozyme folds sequentially in three steps from the unfolded to the native state (Fig. 1B), with a previously uncharacterized on-pathway folding intermediate, extending earlier studies that suggested a folding pathway devoid of kinetic traps. High [Mg$^{2+}$] (>10 mM) is required in vitro to populate the near-native state F significantly. We propose that the newly discovered N state corresponds to the active state, but it can only be distinguished from F above 20 mM Mg$^{2+}$. Because N is a transient conformer in the absence of substrate, it was not observed in bulk experiments.

We observe no Mg$^{2+}$ dependence for the I to F transition (Figs. 4 and 6D), similar to the *Tetrahymena* ribozyme (29) and the RNase P C domain (30). A possible explanation is that this step is dominated by a conformational capture mechanism, in which the F conformation is unstable and must be trapped by Mg$^{2+}$ coordination. A recent study showing that the rate of D1 compaction does not depend on Mg$^{2+}$, whereas the compaction amplitude does (20), supports this idea. Hydroxyl radical footprinting experiments also show that D5 becomes fully protected only above 100 mM Mg$^{2+}$, in agreement with our observations (14). Mg$^{2+}$ ions are, therefore, essential to capture D3 and D5 in their active conformation and to stabilize the D135 complex in vitro. Interestingly, we observed increased RNA structural dynamics upon Mg$^{2+}$ binding (Fig. 6A), linking domain motion to catalysis.

The relative stability of the F and N states remains constant above 40 mM Mg$^{2+}$, ruling out a capture mechanism involving Mg$^{2+}$ for this step. Instead, substrate binding stabilizes the N state, making it the catalytically active species (Fig. 5). The FRET increase from F to N indicates that a major conformational change accompanies D135 compaction. Because the IBS-EBS and e-e' interactions are located within D1 and thus, cannot be observed with our labeling scheme, it must be the $\lambda - \lambda^*$ interaction, which links the intronic 5' end with central nucleotides in D1 and the catalytically crucial D5 (31), that causes this major rearrangement of domains. A recently published crystal structure of a group IIB intron further supports our findings (32). This structure shows that the product form of the RNA folds into a highly compacted state where domains 1-4 interact with the catalytic domain 5. We propose that formation of this highly compacted state might correspond to our F to N transition. However, a quantitative structural comparison with our data is not possible because the loops where the fluorophores are attached are not present in the crystallized group II intron from *Oceanobacillus iheyensis*. In addition, there is no structural information available on the loop where the donor is located, which could easily introduce an error of 10-20 Å on any distance estimate from the crystal structure.

The transient nature of the native state raises the question of whether the rarely occurring transition from F to N, regulated by $k_2$, is limiting for cleavage. To address this question we measured the cleavage kinetics of the ribozyme at room temperature and 100 mM Mg$^{2+}$. We found a cleavage rate $k_{obs} = 0.024 \pm 0.001$ min$^{-1}$ (see Fig. S7), 180-fold slower than $k_2$. This suggests that the transition from F to N alone is not rate-limiting. Alternatively, a combination of folding, catalysis, and activation of the static population may be rate-limiting for group II intron catalysis. Testing this hypothesis will be the scope of future work.

**Implications for Group II Intron Folding and Function in Vivo.** Our results show that large multidomain RNAs, such as D135–L14, can fold in a highly ordered stepwise pathway consisting only of on-pathway intermediates without kinetic traps. This strategy is very advantageous for a retroelement such as a group II intron that must fold cotranscriptionally in vivo. It allows folding to start in D1, which is transcribed first. Then, the slow transition from U to I ensues proper folding of this domain and subsequent docking of the downstream domains as soon as they are transcribed (e.g., D3 and D5).

Although Sc.ai5γ requires high salt and temperature to function in vitro, Ms.s116, a DEAD-box ATPase with helicase activity, promotes splicing in vivo (33, 34). It has been a matter of debate as to whether Ms.s116-unwinding activity is needed to resolve kinetic traps (35, 36). The absence of folding kinetic traps in vitro supports a model where Ms.s116 is needed only to stabilize the native fold at low [Mg$^{2+}$] and not to help the RNA escape from misfolded intermediates.

**Materials and Methods**

**Preparation of RNA.** The D135 ribozyme sequence from *S. cerevisiae* intron Sc.ai5γ on plasmid pT7D135 was modified by insertion of two loops with the length of 18 nt each. Sequence L1 (3′-CCCCAAUAUAACCGUCUUGGUAAGG-5′) was placed between residues 276 and 305 on the d2b stem of D1 (Fig. S1), and L2 (3′-CCCCAAUAUAUUGCCAGACUAGGGS-5′) replaced the residues 681–806 in D4, giving the plasmid pT7D135-L14. Digestion with HindIII and subsequent *in vitro* transcription with home-made T7 RNA polymerase under standard conditions (23) gave D135–L14 RNA, which was stored in water at $-20^\circ$C.

Biotinylated DNA strands, Cy3-DNA, Cy5-DNA, and the RNA substrates 17/7 (3′-UGCCGAGA(dC)UUUUACAGGGUGGUGC-5′) and 17/7-dC (3′-UGCCGAG(dC)UUUUACAGGGUGGUGC-5′, 2′-protected) were purchased from Howard Hughes Medical Institution Biopolymer/Keck Foundation Bio-technology Resource Laboratory (Yale University, New Haven, CT). DNA

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strands were purified by denaturing 18% PAGE and subsequent C8 reversed-phase HPLC as described in ref. 37. The RNAs were deproteinized as suggested by the manufacturer and subsequently gel- and HPLC-purified (37).

Design of a Fluorophore-Labeled Group II Intron Ribozyme for Single-Molecule Studies. The D135 ribozyme is a 618-nt-long single-stranded RNA (Fig. S1) (16, 17), which makes it highly impractical to label using a synthetic approach. Hence, we introduced two modular loops within the D135 sequence at locations within D1 and D4 dispensable for catalysis (9) as described (Fig. 1 A and Fig. S1) (21). The resulting construct D135–L14 contained all domains necessary for catalytic function of the intron and an expendable 37-nt extended 3′ tail (Fig. 1 A). Two DNA-oligonucleotides specifically complementary to each extended loop in D1 and D4 carried the FRET pair Cy3 (Cy3-DNA) and Cy5 (Cy5-DNA), respectively. In addition, a biotinylated DNA-oligonucleotide that hybridizes specifically to the 3′ end tail of D135–L14 was used for surface immobilization in the single-molecule experiments (Fig. 1 A). D135–L14 binds all three DNA-oligonucleotides as expected (Fig. S2).

Steady-State FRET Kinetic Assay. Ratios of Cy3-DNA to ribozyme to Cy5-DNA were chosen to be 1:2.5:6 to minimize unbound amount of Cy3-DNA and maximize Cy5 binding to ribozyme for optimized fluorescence transfer. Cy3-DNA (0.1 μM), D135–L14 (0.25 μM), and Cy5-DNA (0.6 μM) were denatured at 90°C for 1 min in reaction buffer and subsequently incubated at 42°C for 15–20 min. Reaction mixture (100 μl final) was excited at 555 nm, and emission was recorded at 655 nm and 665 nm on a Cary Eclipse fluorescence spectrometer. Slits were set at 5 nm, and sample was thermostatted at 42°C. Emission time courses were measured for 15 min after the addition of varying amounts of MgCl2 in the range of 0–100 mM.

Normalized absolute FRET changes (FRET(0) − FRET(t)) were plotted against Mg2+ concentrations for analysis of overall Mg2+ dependence of folding by the Hill equation (12). Time courses of FRET ratio (defined as E(t) = [FRET(t)]/[FRET(0)]) were fitted with a single exponential expression.

Single-Molecule FRET Experiments. Cy3-DNA, Cy5-DNA, and T-biotin-DNA (10 μM each) were heat-annealed to D135–L14 (1 μM) in reaction buffer containing 0.5% mercaptoethanol. After the addition of MgCl2 (0–100 mM final), the reaction mixture was incubated at 42°C for 15–20 min. The FRET-labeled, biotinylated, and annealed ribozyme complex was diluted to ~25–50 pM and bound to a streptavidin-coated quartz slide surface. Excess Cy3-DNA, Cy5-DNA, and T-biotin oligonucleotides were removed from the slide by a washing step with reaction buffer. An oxygen-scavenging system consisting of 10% (v/v) glucose, 2% (v/v) mercaptoethanol, 50 μg/ml glucose oxidase, and 10 μg/ml catalase was used in all experiments to reduce photobleaching. All smFRET experiments were carried out at room temperature according to described procedures (25, 27, 38). The donor (I0) and acceptor (I) fluorescence signals of optically resolved single molecules were recorded on a total internal reflection fluorescence microscope as described (27, 38). Donor and acceptor signals were found to bleach in a single step, confirming the presence of single molecules. Single-molecule traces showing dynamics and before photobleaching of typically ~150 molecules at each concentration were manually selected, and E(t) values for individual Mg2+ concentrations were accumulated in histograms. Histogram distributions were analyzed with a double or triple Gaussian equation to reveal reoccurring mean FRET values. Dwell times from single-molecule trajectories were calculated and plotted in dwell time histograms for calculation of rate constants as described in ref. 25. Control experiments in the absence of D135–L14 or biotin-DNA did not exhibit any conformational dynamics, ruling out nonspecific interactions between the label DNA or the RNA and the quartz surface. All RNA systems studied to date exhibit only negligible perturbations from surface immobilization (39).

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