Supplementary Data for

A rugged free energy landscape separates multiple functional RNA folds throughout denaturation

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Supplementary Data Contents:

Supplementary Methods

**Supplementary Figure 1:** Redistribution through strand replacement facilitated by heating.

**Supplementary Figure 2:** Redistribution achieved through strand displacement by a DNA oligonucleotide.

**Supplementary Figure 3:** Folding heterogeneity in a catalytically active trans-cleaving t-2WJ hairpin ribozyme.
SUPPLEMENTARY METHODS

In vitro transcription
Partially double stranded DNA containing a T7 promoter was incubated in buffer containing 120 mM HEPES-KOH pH 7.5, 30 mM MgCl₂, 2 mM Spermidine, 40 mM DTT, 0.01% triton x-100 (v/v) with 4 mM each of NTPs, T7 RNA polymerase, and pyrophosphatase for 3 h at 37° C. Full length transcripts were isolated using denaturing PAGE (20% acrylamide, 8 M Urea). RNA concentrations were calculated from their absorption at 260 nm correcting for absorbance by attached fluorophores when applicable.

EMSA with FRET detection
For FRET gels 1 μM fluorescently labeled strand RzA was annealed in the presence of 2-3 fold excess of unlabeled RzB strand. For the radioactive gel in Fig. 1 RzB was 3'-32P radiolabeled using pCp and T4 RNA ligase and used in trace amount for visualization in EMSA gels prepared with 10 nM labeled RzB and 500 nM unlabeled RzA. For radioactive gels of fully transcribed ribozyme as in Fig. 3 both RzA and strand RzB were 5’-32P-labeled using ATP and T4 polynucleotide kinase and used in trace amounts for visualization in EMSA gels prepared with 6 μM of RzA and RzB. Non-denaturing FRET gels were imaged using FluorImager SI fluorescence scanner and ImageQuant software (Molecular Dynamics). A laser excites fluorescein at 488 nm, and the gels are scanned for fluorescence emission using a photomultiplier tube with either a 530 nm band-pass (for the donor fluorescein) or a 610 nm long-pass filter (for the acceptor tetramethylrhodamine). With the readout of donor defined as green and that of acceptor as red, the corresponding color images were superimposed using Photoshop 5.5 as described previously (5). Radioactive gels were imaged with either PhosphorImager screens or autoradiography. Quantification of native radioactive gel distributions was achieved by fitting to two Lorentzian distributions as described (6).

Single molecule FRET Dwell Time Analysis
Dwell times for the docked and undocked states were analyzed and fit as described previously (2, 7, 8). For the multiple-turnover measurements with cleavable ribozyme 660 dwell times in the docked state, and 682 dwell times in the undocked state from 123 smFRET time trajectories measured at 500 mM MgCl₂ were used for the fits. We note that increasing ionic strength has previously been shown to accelerate docking while having essentially no impact on undocking kinetics (7), thus increasing the number of events observed for each molecule. The rate constants and corresponding amplitudes for docking and undocking of the non-cleavable ribozyme used for the EMSA separation were determined by performing a global fit on all three normalized data sets (T, B, and unseparated), holding the rate constants constant across all data sets and allowing all other parameters to vary independently throughout the fit. A total of 829 dwell times in the docked state and 831 dwell times in the undocked state from 295 single-molecule FRET time trajectories were used in the global fit. In contrast to previous work, we observe two docking rate constants, however the two docking rates appear to be independent of the undocking rates and only differ by approximately one order of
magnitude (or less in the case of the non-cleavable sample). We therefore determined the time-window corrected fraction of ribozymes undocking with each of the undocking rate constants separately for each of the docking rates, as described previously (2), and used the weighted average of those results to determine the final fractions given in Figure 3d, Figure 1c, and Figure S3. Please note that, after time window correction, the molecule fractions are no longer identical to the amplitudes from the exponential fits due to the statistical underrepresentation of slow events, as previously described (2).

Footprinting
Footprinting gels were quantified using a PhosphorImager Storm 840 with ImageQuant software. The concentration of material was determined by boxing with ImageQuant. In order to normalize cleavage values and account for differences in background cleavage Π values were determined using the equation as described (10):

$$\Pi = \frac{\sum_{i} \text{band intensity at nucleotide } x_{i}}{\sum_{i} \text{band intensity at nucleotide } i}$$

where $x$ is the analyzed nucleotide position and 0 mM [Tb$^{3+}$] is a control reaction incubated in the same manner as the Tb$^{3+}$ containing reaction, except without the addition of Tb$^{3+}$. The ratio between Π values of individual nucleotides in the T and B species was determined for comparison. Final Π ratios are the result of at least two independent footprinting reactions. Π value ratios $>2$ or $<0.5$ are considered to indicate a significant difference between the two species.

DNA-Assisted Strand Displacement
Following EMSA separation, the ribozyme was eluted from the EMSA gel into a buffer containing 50 mM Tris-HCl (pH 7.5), and 12 mM MgCl$_2$. The separated material was then incubated for 2 h at 37°C with $>10$ fold excess of a DNA oligonucleotide fully complementary to one of the RNA strands. Strand invasion by the DNA oligonucleotide results in base pairing between the DNA and the complementary RNA strand (RzA or RzB depending on the DNA oligonucleotide used), efficiently separating the two ribozyme stands. This process results in one of the stands being incorporated into a fully base-paired DNA-RNA hybrid, while the other strand is converted into single-stranded form. Excess DNA oligonucleotide was then degraded by incubating with DNase RQ1 for 1 h. Finally, the ribozyme was reformed by replacing the RNA strand sequestered in the DNA-RNA hybrid with fresh strand.

Thermal Denaturation
A volume of 300 μl of annealed ribozyme at 1 μM concentration in standard buffer containing 50 mM Tris-HCl (pH 7.5), and 12 mM MgCl$_2$ was degassed followed by thermal denaturation. The sample was heated at 0.2°C/s from 35-99°C and UV
absorbance at 260 nm was monitored using Beckman DU-640B UV–Vis spectrophotometer. Melting temperatures were determined from two separate measurements by fitting the first derivative of the melting curves to two Gaussian distributions.
Figure 1. Redistribution of the fluorescein and tetramethylrhodamine doubly labeled T and B species through heat-assisted RNA strand replacement. (a, b) Redistribution of the T and B species in a background of either excess RzA (lane XS-A) or RzB (all other lanes except for control lane) as monitored by EMSA. Isolated T species (a) or B species (b) was heated to the indicated temperature for 2 min and then cooled to allow for reannealing with the excess RzA (or RzB) strand. An analytical EMSA gel was then run at 4°C and its fluorescence detected using a FluorImager SI fluorescence scanner as described above. (c, d) Redistribution of the T and B species in the presence of excess RzB as monitored by tr-FRET. Both species are shifted to similar distance distributions after heating to 95°C and then cooling back down to 25°C. The unheated controls (25°C, in color) are shown for reference. (e) Heating temperature dependence of the apparent docking equilibrium constant $K_{dockapp} = \frac{\text{fraction(docked)}}{\text{fraction(undocked)}}$ derived from tr-FRET measurements similar to those described in panels c and d. Two Gaussian distance distributions were fit to the tr-FRET data to determine the fractions of docked (smaller distance) and undocked conformation (larger distance) after heating the T (triangles) and B species (squares) in the presence of excess RzB to the indicated temperature. (f) UV-detected melting curve of unseparated hairpin ribozyme with apparent melting temperatures indicated. The UV-detected melting transitions, indicative of loss of secondary structure, coincide with interconversion of the T and B species in the presence of excess RzB (panel e), consistent with the notion that interconversion occurs via strand replacement.
Figure 2. Redistribution of the isolated fluorescein and tetramethylrhodamin doubly labeled T and B species as achieved through DNA-assisted strand displacement. DNA oligonucleotides fully complementary to either RzA (lanes with D(A)) or RzB (lanes with D(B)) were used to disrupt the ribozyme’s secondary structure by base pairing with their complement through strand invasion. This process results in the complementary RNA strand fully incorporating into a DNA-RNA hybrid, whereas the other RNA strand becomes single-stranded. Addition of either fresh RzA or RzB strand, as indicated for the corresponding lanes, was then used to reanneal the ribozyme and the result analyzed as described in Supplementary Methods. The EMSA and FluorImager analysis shows that partial redistribution between the T and B species occurs under these conditions, but only in the presence of fresh RzA or RzB strand. Lanes T and B contain the isolated T and B species, respectively, and the Control lane shows the unseparated hairpin ribozyme.
Figure 3. Folding heterogeneity in the catalytically active trans-cleaving t-2WJ hairpin ribozyme. Cumulative number of dwell times (N) shorter than time t in the (a) undocked and (b) docked states, and (c) in the substrate-free state (after product release and before renewed substrate binding) are plotted for the t-2WJ ribozyme under multipleturnover conditions at high ionic strength (500 mM MgCl₂). Solid red lines indicate the (multi-)exponential fits used to determine, as described in Supplementary Methods, the docking (a), undocking (b), and substrate binding rate constants (c) with their time-window corrected fractions reported in the table insets. (d and e) Examples of smFRET trajectories displaying distinct undocking behaviors in the presence of 12 mM and 100 mM MgCl₂, respectively.
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