Full Methods

Reagents

Enzymes: T7 RNA polymerase (His-tagged) was over-expressed in BL21 DE3 cells and purified by chromatography on Ni$^{2+}$-resin, followed by dialysis against a storage buffer containing 20 mM phosphate buffer (pH 7.5), 100 mM NaCl, 10 mM DTT, 1 mM EDTA and 50% glycerol. Glucose oxidase (Type VII from *Aspergillus niger*, Sigma-Aldrich) and catalase (Roche Applied Science) were used as supplied.

Chemicals: D-glucose (SigmaUltra, 99.5%), guanosine 5′-monophosphate (Sigma-Aldrich, ≥99%), dNTPs (Fermentas, >98%), NTPs (Sigma, 95-99%), Trolox® ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Aldrich, >97%), egg lecithin (Avanti Lipids) and cap-biotin phosphatidylethanolamine (PE, Avanti Lipids) were used without further purification.

Ribozyme preparation

L-16T2 ribozyme, which is a version of the L-21 Scal ribozyme extended at the 5′-end by a sequence GGUUU and at the 3′-end by the sequence ACCAAAAUCAACCUAACACUUACACA, was prepared by PCR amplification of a DNA template from a plasmid pT7L-21 with the extensions encoded on the primers. The ribozyme was prepared by a run-off *in vitro* transcription of the DNA template in conditions that minimize self-processing of the 5′-end (30 min at 30°C with 4 mM MgCl$_2$ present). RNA was purified on a denaturing PAGE (8% of 29:1 acrylamide:bis-acrylamide) and stored as a ~20 μM stock solution at –20°C.
**Oligonucleotide labeling**

RNA oligonucleotides CCCUCdUA,\(^1\) later referred to as (-1d)S,  
CCCUCmUAAACC\(^2\) referred to as (-1m)S, and CCCmUCUAAACC referred to as (-3m)S, with 3′-amino modification were purchased from Dharmacon (now Thermo Scientific) and deprotected according to manufacturer’s instructions. 3′-Amino groups were modified with Cy3-NHS (Invitrogen™) and labelled oligonucleotides were purified from unlabelled and shorter oligonucleotides (present in synthetic oligonucleotides because of <100% coupling efficiency) by denaturing PAGE (20% of 29:1 acrylamide:bis-acrylamide). After purification oligonucleotides were dissolved in distilled water and stored at –80 °C.

DNA oligo T2b (TGTGTAAGTTTTAGGTTGATTTTGGT) with 5′-biotin and 3′-amino modifications was purchased from Integrated DNA Technologies Inc., labeled with Cy5-NHS and purified as described above.

**Sample preparation**

Solutions of L-16T2 ribozyme and T2b-Cy5 were mixed together (2:1 molar ratio, final concentration of RNA 1 µM) in an annealing buffer (50 mM NaMES, pH 6.0, 200 mM NaCl) and annealed by heating at 95 °C for 3 min, then cooling to 50 °C at 0.1 °C /s. Folding was initiated by adding MgCl\(_2\) to a final concentration of 10 mM. The ribozyme was folded for 30 min at 50 °C.\(^{30}\)

Oligonucleotide substrates were bound by mixing 2 µl of pre-folded L-16*T2b-Cy5 (1 µM) and 0.5 µl of S-Cy3 (2 µM) in 50 mM NaMES (pH 6.0) and 10 mM MgCl\(_2\).

\(^1\) dU stands for 2′-deoxyuridine  
\(^2\) mU stands for 2′-methoxyuridine
and incubating at room temperature for 10 min. Ternary complex L-16*T2b-Cy5*S-Cy3 was diluted to a final concentration of 30-100 pM for deposition on the slides.

Quartz slides (G. Finkenbeiner Inc.) were coated with biotinylated bovine serum albumin (Sigma, 1 mg/ml for 10 min), washed extensively with 50 mM NaMOPS (pH 7.0), then coated with streptavidin (Sigma, 0.1 mg/ml for 10 min), washed extensively with 50 mM NaMOPS (pH 7.0) and in the end with 50 mM NaMES (pH 6.0) and 10 mM MgCl₂.

Ternary complexes L-16T2/T2b-Cy5/S-Cy3 were deposited over the coated slides for 10 min followed by extensive washing with 50 mM NaMES and 10 mM MgCl₂. Measurements of docking were performed in a standard buffer containing 50 mM NaMES (pH 7.0), 10 mM MgCl₂, 100 mM NaCl and an oxygen scavenging system (100 units/ml glucose oxidase, 1000 units/ml catalase Biosciences, 10 mM D-glucose and 2 mM Trolox). Cleavage buffer was the same, except for using 50 mM NaMOPS (pH 8.1) instead of NaMES, and adding 1 mM guanosine 5′-phosphate (Sigma). Higher pH accelerates cleavage, thereby facilitating measurement of the chemical step, and has been shown to have no effect on docking.³⁰

**TIRF microscope and data acquisition**

A green laser (532 nm DPSS, “Gem” by Laser Quantum) and a 635 nm red laser (Hitachi HL6344G diode, max power 10 mW) beam were combined using dichroic mirrors and focused through a prism onto a sample contained in a flow cell made from a quartz slide and a cover slip glued together by a double sided tape. Laser beams enter the prism at an angle ensuring total internal reflection of the exciting light. Images were collected by a 60x water immersion Nikon objective (NA 1.2), filtered through a 550 long pass filter (Chroma Technology) to remove scattered excitation light, and
chromatically separated by dichroic mirrors (635 nm cutoff) into a “green” image and a “red” image. The “green” image, filtered through a 580/30 band pass filter, and the “red” image, filtered through 670/30 band pass filter, were focused, respectively, onto the left and right halves of a back-illuminated charge multiplying CCD (Cascade128+ camera by Photometrics®, Roper Scientific Inc). Full CCD images (128×128 pixels) were read out in 40 ms frames with a conversion gain 3 and multiplication gain 3500. The red laser was typically switched on only for the first 0.5 s of data acquisition, and at the end of data acquisition to determine which of the molecules had fluorescently active Cy5 dye. The intensity of the green laser was adjusted to achieve an average signal-to-noise ratio >5, which typically required ~20 mW power at the laser aperture.

Data analysis and simulations

Recording FRET data. Positions of spots of interest within each data acquisition “movie” were determined by averaging the first 30 frames and finding pixels on the “red” side of the CCD with an intensity that exceeds a certain threshold (typically, threshold = 5σ, where σ is the standard deviation of the background fluorescence). The corresponding positions on the “green” side were determined by applying linear offsets (determined independently from images of fluorescent beads that are visible on both sides of the CCD) and further refining by the “affine” algorithm of Matlab. Local (7×7 pixel) background was subtracted from each spot for each frame. Time traces of fluorescent intensity of Cy3 and the Cy5 for each spot were recorded and used to calculate a FRET trace (for each frame, $FRET = I_{\text{red}}/(I_{\text{green}} + I_{\text{red}} - I_{\text{Crosstalk}})$. The value of the cross-talk (intensity in the red channel arising from Cy3 fluorescence because of imperfect chromatic separation) was determined by measuring the “red” intensity for molecules that contain only Cy3.
Identifying molecules. For each spot, Cy 3 and Cy5 traces were visually screened to determine if they corresponded to a single RNA molecule, and accepted if they met the following criteria:

1) Single step photobleaching (fluorescence intensity goes to the background level within one frame);

2) Stable signal (average fluorescence intensity along the trace is constant; it does not gradually decrease or increase);

3) Normal signal strength (fluorescence intensity is within 2-fold of the average; very dim and very bright spots were rejected);

4) Cy5 is fluorescent (“red” fluorescence is detected when the red laser is switched on at the beginning and the end of data acquisition; if Cy5 was fluorescent at the beginning, but not at the end of data acquisition, the trace was truncated at the last frame where Cy5 was fluorescent).

Measuring thermodynamics and kinetics of individual molecules. FRET traces for >95% of molecules (defined by the above criteria) displayed fluctuations between two FRET levels, low FRET = 0.35 and high FRET = 0.95, corresponding to undocked and docked state, respectively, as described previously. Transitions between FRET levels were identified by a standard thresholding method. The threshold was set at 0.7. On average, each trace contained ~ 30 transitions, allowing precise estimates of the rate constants. Rate constants were determined by creating histograms of dwell times in the docked state (to measure $k_{\text{dock}}$) and in the undocked state (to measure $k_{\text{undock}}$), and fitting histograms to a sum of exponentials. For most traces, the histograms were well described by a single exponential decay. Equilibrium constants between docked and undocked conformations were determined as a ratio of the total time spent
in docked ($t_{\text{dock}}$) and in undocked ($t_{\text{undock}}$) states, $K_{\text{dock}} = t_{\text{dock}}/t_{\text{undock}}$ and, from it, the free energy of docking was calculated as: $\Delta G_{\text{dock}} = -RT \times \ln K_{\text{dock}}$.

Because of photobleaching, each molecule can be observed for only a limited period of time, $t_{\text{life}} \sim 1/k_{\text{bleach}}$, which makes $\Delta G_{\text{dock}}$ calculated for each trace a random parameter distributed around a true mean. For a trace that fluctuates between two FRET levels with a forward rate constant $k_{\text{dock}}$ and a reverse rate constant $k_{\text{undock}}$, the width of the $\Delta G_{\text{dock}}$ distribution depends only on $k_{\text{dock}}$, $k_{\text{undock}}$ and $k_{\text{bleach}}$. To estimate the extent of broadening, we first measured the distribution of trace lengths, and from it calculated the photobleaching rate constant $k_{\text{bleach}} = 0.03 \text{ s}^{-1}$ (Fig. S1). We then performed numerical simulations of the $\Delta G_{\text{dock}}$ distribution for a broad range of $k_{\text{dock}}$ and $k_{\text{undock}}$ values, keeping $k_{\text{bleach}}$ fixed. For each pair of parameters $k_{\text{dock}}$ and $k_{\text{undock}}$, 1000 traces were simulated by a lab-written program in Matlab (Mathworks Co.), $\Delta G_{\text{dock}}$ for each trace was calculated as above, and histograms were fit by a Gaussian distribution. As the results of the simulations demonstrate (Fig. S2), for any combination of the rate constants $k_{\text{dock}}$ and $k_{\text{undock}}$ that were experimentally measured, the width ($w = 2\sigma$, where $\sigma$ is the standard deviation) of the $\Delta G_{\text{dock}}$ distribution is small (typically, $\leq 0.3 \text{ kcal/mol}$) compared to the experimentally observed width ($\sim 1 \text{ kcal/mol}$). Comparing the results of these simulations with the experimental data, it is easy to see that a single population of molecules, characterized by a single $\Delta G_{\text{dock}}$, $k_{\text{dock}}$ and $k_{\text{undock}}$, cannot account for the experimentally observed width of the $\Delta G_{\text{dock}}$ distribution. As is illustrated in Fig. S3, at least six distinct populations, each with different $\Delta G_{\text{dock}}$, $k_{\text{dock}}$ and $k_{\text{undock}}$, are required to account for the full width of the distribution. These six conformations should be considered a minimum number of co-existing conformations that can be determined with limited resolution of $\Delta G_{\text{dock}}$ that is imposed, ultimately, by photobleaching.
Cleavage experiments

First, docking was observed for 20 s essentially as described above. Then, the laser was switched off and the cleavage buffer containing 1 mM guanosine 5′-phosphate was flowed through the sample at ~40 μl/s for 5 s. Immediately after buffer replacement, and then subsequently after several time intervals, the laser was switched on for 2 s at a time (to allow confident detection of remaining molecules, while limiting photobleaching) and images of the slide were recorded. Fluorescent spots were defined as molecules with the substrate uncleaved if they fulfilled the criteria of signal stability and intensity (#2 and #3 in “Data Analysis” above). Control experiments establishing that the rate of substrate disappearance in the absence of guanosine is negligibly small were performed in exactly the same way, but omitting guanosine 5′-phosphate from the cleavage buffer.

Unfolding/refolding experiments

The experiments were done in two steps: in the first step, docking was observed for 20 s essentially as described above. Then the laser was switched off and the unfolding buffer containing 12 mM EDTA was flowed through the sample at ~40 μl/s for 5 s. Unfolding was ensured by quickly switching the laser on and verifying that all molecules were in low FRET state. After 1 min in EDTA, the standard folding buffer was flowed back in and docking was observed for 20 s. Docking equilibria for each molecule before and after unfolding were measured as described above. To test whether molecules change their docking behavior, the uncertainty of measuring the docking equilibrium was calculated as the width of the simulated distribution (Fig. S2). The p-values were calculated using right-tailed chi-square variance test implemented in Matlab testing the null hypothesis that the difference between docking equilibrium before and after unfolding is equal to, or smaller than, the uncertainty of the measurement.