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SINGLE-MOLECULE RNA SCIENCE

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■ **Abstract** The development of single-molecule detection and manipulation has allowed us to monitor the behavior of individual biological molecules and molecular complexes in real time. This approach significantly expands our capability to characterize complex dynamics of biological processes, allowing transient intermediate states and parallel kinetic pathways to be directly observed. Exploring this capability to elucidate complex dynamics, recent single-molecule experiments on RNA folding and catalysis have improved our understanding of the folding energy landscape of RNA and allowed us to better dissect complex RNA catalytic reactions, including translation by the ribosome.

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INTRODUCTION

This is not the first time that we have witnessed RNA taking center stage. Some 20 years ago, two independent, groundbreaking discoveries changed our view about this biomacromolecule: Found to function as catalysts, RNA molecules could

no longer be viewed as merely messengers that pass information from the DNA to proteins (19, 28). Since then, a growing number of essential reactions in cells are have been found to be catalyzed by RNA enzymes. Two examples exist at the heart of the central dogma of biology: The ribosome, the giant ribonucleoprotein assembly responsible for protein synthesis in cells, uses its RNA components to perform major catalytic functions (12, 37, 38). The spliceosome also likely uses its RNA constituent to catalyze the splicing reaction of the precursor RNA (70, 71), while the 100 or so protein splicing factors regulate the reaction. In addition to these essential catalytic functions, we now witness a second revolution in the world of RNA: its remarkable regulatory role. In the past decade or so, hundreds of small noncoding RNAs, microRNAs, and endogenous small-interfering RNAs were discovered that regulate gene expression either by inducing messenger RNA degradation or by suppressing translation in both prokaryotic and eukaryotic cells (3, 11, 17). Aside from these posttranscriptional regulations, gene-regulation by noncoding RNA at the transcription level has also been observed recently (17, 74). In keeping with this expanding list of fundamental biological roles, RNA also finds exciting applications in modern biotechnology and medicine, as gene regulatory tools, as therapeutic agents, and in drug discovery (9, 14, 60, 64).

Although RNA science is attracting a tremendous amount of attention, single-molecule studies of RNA are also gaining momentum. There are good reasons for this to happen. With a growing appreciation of the importance of RNA, a mechanistic understanding of how RNA molecules adopt specific structures that mediate their functions is becoming more desirable than ever. Research on this RNA folding problem suggests that the free energy landscape of RNA is often highly rugged (8, 24, 41–43, 45, 46, 50, 52–54, 56, 66, 67, 69, 78–82). As a result, an RNA molecule can traverse a multitude of kinetic paths and intermediate conformational states before attaining its native structure. For example, oligonucleotide hybridization, hydroxyl radical footprinting, circular dichroism, and UV absorption assays have led to the discovery that RNA folding occurs through a series of intermediate states (46, 56, 69, 79, 80). RNA enzymes were also seen to fold along multiple pathways by native gel electrophoresis and enzymatic activity assays (41–43, 52). However, these methods that measure the average property of an ensemble of molecules can only probe accumulative intermediate states and significantly populated pathways; they cannot detect nonaccumulative, transient intermediate states. The existence of a large number of folding pathways will also make the characterization of each individual pathway difficult by ensemble assays. In these cases, single-molecule approaches come to the rescue owing to their intrinsic capability to detect nonaccumulative transient intermediate states and heterogeneity in the system.

Similarly, the reactions catalyzed by RNA enzymes, especially those by large ribonucleoprotein complexes such as the ribosome, tend to be complex, involving many reaction steps and reaction intermediates. These steps are typically not synchronized for an ensemble of molecules, making the reactions difficult to dissect. Although stalling the reaction complexes at specific intermediate states has

provided us with a lot of molecular insights into these reactions, this kind of manipulation is not necessarily possible for every intermediate state. Monitoring the reaction of a single molecule or molecular complex naturally overcomes this synchronization problem, offering us an opportunity to resolve each microscopic step of a complex reaction.

The power of single-molecule techniques to elucidate complex dynamics does not come without sacrifices. Because of the limited amount of signal that a single molecule can produce, the time and spatial resolutions of single-molecule experiments are relatively limited compared with ensemble experiments. Luckily, RNA folding and catalytic reactions are not so fast, typically occurring on the timescales of milliseconds to minutes. In addition, these reactions often involve large conformational changes. These properties allow current, state-of-the-art single-molecule experiments to explore the RNA world. Indeed, these experiments have already made significant contributions to our understanding of the structural biology and enzymology of RNA. In this article, I review some of the recent developments in RNA science made possible by single-molecule techniques, focusing on those by fluorescence resonance energy transfer (FRET) and optical tweezers.

EXPERIMENTAL TECHNIQUES

Single-Molecule FRET

FRET is a powerful assay to measure the intra- and intermolecular motions of biomolecules in real time (57, 63, 75). In this assay, a pair of fluorescent donor and acceptor molecules is attached to the host biomolecule(s) of interest. The dipole-dipole interaction between the donor and acceptor causes energy transfer between them, leading to a decrease in the donor emission and an increase in the acceptor emission. The energy transfer efficiency E is given by $E = 1/(1 + (R/R_0)^6)$, where R is the distance between the donor and the acceptor. The Förster radius R_0 is typically 3 to 8 nm, making FRET sensitive to changes of a few nanometers in the donor-acceptor distance, which can reflect either the conformation changes of the host molecule or relative motions between two molecules. The dependence of energy transfer efficiency on the orientation of the donor and acceptor fluorophores, however, introduces uncertainty into FRET-based distance determinations. This problem can be mitigated if the dyes are attached to the host molecule with flexible linkers to ensure their free rotation. As this condition is difficult to satisfy rigorously in practice, researchers should be cautious when translating FRET efficiencies into absolute distances quantitatively.

The development of single-molecule fluorescence spectroscopy (33, 36, 77) has allowed FRET to be measured at the single-molecule level (21, 55). Here, I briefly discuss a few aspects of single-molecule detection that are critical for unveiling the dynamic information of biomolecules. Like all single-molecule fluorescence techniques, efficient reduction of the background signal is critical

for single-molecule FRET. This is often achieved by confocal detection or by excitation with an evanescent wave generated by total internal reflection. The total internal reflection microscope allows a whole field of molecules, typically several hundred of them, to be detected simultaneously. Such a parallel detection greatly facilitates the building of statistics, especially for irreversible processes. However, wide-field detection requires the use of cameras, which limit the data acquisition rate to 1 kilocycle per second or slower using state-of-the-art charge couple device (CCD) cameras with on-chip amplification. Higher data acquisition rates, up to tens of megacycles per second, can be achieved with confocal microscopes using sensitive point detectors, such as avalanche photodiodes or photomultipliers.

Besides physical instrumentation, special manipulation of biomolecules is often required for real-time characterizations. With a confocal microscope, it is possible to detect freely diffusing molecules in solution (13, 36). Although this detection geometry avoids any potentially adverse effect arising from the confinement of molecules, the range of timescales accessible by this method is severely limited by the dwell time of a molecule in the confocal detection volume, typically in the submilliseconds governed by diffusion. Therefore, to expand the dynamic range of the single-molecule experiments, various strategies were developed to confine the biomolecules of interest within the detection volume of a microscope. The two most commonly used strategies are confinement by matrices, such as agarose or polyacrylamide gels (32), and immobilization to surfaces with specific interactions, such as via the biotin-streptavidin bridge (22). The latter method is compatible with rapid buffer exchange and therefore is better suited for characterizing reactions. Although surface immobilization may perturb the biomolecules, this perturbation has been rather insignificant for most of the RNA systems studied to date, at neutral or basic pH values. For systems that involve proteins, which tend to interact strongly with surfaces through hydrophobic interactions, surface passivation is often required to retain the integrity of the system. Polyethylene glyco (PEG) is a commonly used passivating reagent (34). Another method is to entrap the biomolecule in a lipid vesicle attached to a surface (48). This technique potentially offers a close-to-physiological environment for the biomolecule; however, a method for rapidly changing the buffer in the vesicle needs to be developed before this method can be broadly used for characterizing biological processes.

With the molecules confined in the detection volume, dynamics can be probed at the single-molecule level over a wide range of timescales, ranging from millisecond to minutes or even hours. The lower limit is imposed by the photon emission rate from a single fluorophore under conditions in which photoblinking or photobleaching is not severe enough to render the experiment impractical. The recent development of CCD cameras with on-chip amplification allows this limit to be achieved even in the wide-field-detection geometry. This limit can be further improved by using fluorescence correlation spectroscopy if the process under investigation is reversible and if the forward and backward reaction occurs

spontaneously (25, 27, 76). In this case, the time resolution is limited by the response time of the detectors, which is of the order of tens of nanoseconds for avalanche photodiodes or photomultipliers.

Single-molecule FRET has been used to study a variety of biological systems. In this review, I only cover its application to RNA systems (4–7, 22, 35, 47, 51, 54, 67, 78, 81, 82) but refer interested readers to a number of recent review articles for other applications (20, 75, 83).

Optical Tweezers

Optical tweezers rely on a tightly focused laser beam(s) to trap a particle in three dimensions and, through redirection of the beam, manipulate the particles (1). Focused light exerts two forces on the particle, the gradient force and the scattering force. When the refractive index of the particle is larger than that of the surrounding medium, the gradient force draws the particle toward the focus of the beam, where the light field is the strongest. The scattering force arises from the radiation pressure exerted on the particle by photons that are either absorbed or scattered. When balanced, these two forces hold the particle just slightly downstream of the light focus.

Optical tweezers allow the manipulation and detection of single biomolecules. As biological molecules are often too small to be manipulated directly by optical tweezers, a micron-sized dielectric sphere is often attached to the molecule of interest to serve as a handle. This allows researchers to manipulate the position of the attached biomolecule and to exert a well-defined force. Both parameters can be determined with exquisite accuracy: The position of the microsphere can be measured to within 0.1 nm, and the force to 0.1 pN (39, 58). Forces well above 100 pN can be achieved with a dual-beam optical tweezers (10).

Since the invention of optical tweezers, this technique has been adopted to study a variety of biological systems. This review focuses on the studies on RNA systems (23, 30, 31, 40). Other single-molecule force spectroscopy techniques, such as those using atomic force microscopes and magnetic tweezers, have also provided a wealth of information on biological molecules (61, 62, 83). These single-molecule techniques are fully expected to make significant contributions to our understanding of RNA systems as well.

SINGLE-MOLECULE FRET STUDIES OF RNA FOLDING

Folding of Large, Multidomain RNA Enzymes

The capability of single-molecule FRET to follow conformational changes of RNA was clear early on. In one of the first applications of single-molecule FRET to biological systems, Ha et al. (22) showed that the ligand-induced conformational changes of an RNA three-way junction could be monitored in real time at the single-molecule level. Zhuang et al. (81) performed the first single-molecule

RNA folding study by using a large, multidomain RNA enzyme, the *Tetrahymena* ribozyme (Figure 1a, see color insert), as a model system. The power of single-molecule FRET was already apparent in this early work. A repertoire of dynamic properties that are difficult to analyze with ensemble experiments, including nonaccumulative folding intermediate states, parallel folding pathways, and equilibrium conformational fluctuations, were directly observed in single-molecule FRET trajectories of the *Tetrahymena* ribozyme (81).

FRET trajectories of individual ribozyme molecules directly reveal folding intermediates (Figure 1b): The addition of Mg^{2+} to trigger folding causes the FRET signal to increase from the unfolded state value of 0.1 to an intermediate value (0.3) before attaining the native value (0.9). Statistical analysis of the folding times of each molecule indicated that the *Tetrahymena* ribozyme folds along at least three distinct pathways, with folding rate constant being 1 s, 1 min, and 0.01 hr, respectively. Kinetic traps exist on these pathways. While the two slower pathways have been observed previously in ensemble measurements, the most rapid pathway was first observed in this single-molecule FRET experiment (81).

In a follow-up study designed to gain a greater understanding of the various pathways (54), folding of the *Tetrahymena* ribozyme was started from distinct regions on the energy landscape by varying the monovalent salt concentration of the prefolding solution. Different monovalent salt concentrations led to dramatically different folding behaviors, each with a distinct folding rate constant and traversing a different set of intermediate states. These results were interpreted as the molecules folding along several discrete channels separated by large energy barriers. Site-specific mutation analysis suggests that these different folding behaviors are caused by the different secondary structures formed under various salt concentrations (54), consistent with previous ensemble experiments (43, 44). Recent results from a hydroxyl radical footprinting assay suggest that high concentrations of monovalent salt induce an extensive amount of tertiary structures in addition to secondary structure changes (66). These preformed tertiary structures may also be partly responsible for the observed changes in the folding kinetics induced by preincubation with monovalent salt.

More recently, FRET has been applied to another large RNA molecule, the catalytic domain of *Bacillus subtilis* RNase P RNA (78). Ensemble characterizations of this enzyme indicate the existence of three conformational states: the unfolded state, the intermediate state, and the native state. However, analysis of the molecules at equilibrium using single-molecule FRET suggest that at least seven different conformations exist at intermediate Mg^{2+} concentrations, as distinguished by their corresponding FRET levels and connectivity between different states. The equilibrium structural dynamics of individual RNase P molecules also shows a high level of heterogeneity.

These single-molecule results indicate that large RNA molecules fold across a highly rugged energy landscape, along a multitude of folding pathways, and through many intermediate folding states. These findings not only corroborate

previous and concurrent ensemble characterizations using various footprinting methods, enzymatic activity assays, gel-mobility measurements, and optical scattering or spectroscopic techniques (8, 24, 41–43, 45, 46, 50, 52, 53, 56, 66, 69, 79, 80), but also reveal new folding intermediates and folding pathways difficult to access by ensemble assays, thereby significantly improving our understanding of RNA folding.

Folding of Small RNA Enzymes

To date, single-molecule studies of small RNA enzymes have concentrated on one type of ribozyme, the hairpin ribozyme (Figure 2*a*, see color insert), although other systems are beginning to be explored. The hairpin ribozyme has been suggested to assume either an extended, undocked conformation or a docked conformation in which the two loop domains, A and B, make tertiary contacts (73). Two forms of hairpin ribozymes have been studied at the single-molecule level: the two-way junction ribozyme, a minimal hairpin ribozyme with high enzymatic activity, and the wild-type ribozyme, which has a four-way junction connecting helix 2 and helix 3 (15, 73) (Figure 2*a*). The first single-molecule experiment was performed on the two-way junction ribozyme (82). Strikingly, the FRET time traces of single molecules show highly heterogeneous undocking kinetics with several different rate constants (Figure 2*b,c*). Furthermore, the molecules retain their particular undocking rates through many docking-undocking cycles, and conversion between different undocking behaviors requires at least several hours (Figure 2*b*). These results suggest the existence of rather large numbers of conformational states that are all relatively stable under functional conditions, which is remarkable for such a small RNA enzyme.

The heterogeneous undocking kinetics appear to be ubiquitous to all hairpin ribozymes. For the two-way junction ribozyme, both the wild type and variants with significantly different docking and undocking rate constants show heterogeneous undocking behavior (7, 51, 82). In addition, heterogeneous docking kinetics are seen in a variant with a modification at the domain junction (51). A study on the four-way junction hairpin ribozyme, a construct that more closely resembles the natural form of the ribozyme, also shows pronounced heterogeneity in both docking and undocking kinetics (67).

The comparison between the single-molecule studies of the two- and four-way junction ribozymes reveals an interesting phenomenon: The latter form folds (docks) two to three orders of magnitude faster (67). Evidence suggests that the dramatic enhancement in the folding rate is a result of the intrinsic structural dynamics of the four-way junction: The ribozyme appears to fold into its native form via an intermediate state in which the four-way junction juxtaposes the loop domains, A and B, into proximity but without substantial loop-loop interactions (67). This result is further supported by a more recent single-molecule study of hairpin ribozymes freely diffusing in solution (47). There, even when the two loops are replaced with perfectly base-paired helices, these helices are still juxtaposed

into proximity by the four-way junction, whereas both two-way and three-way junctions lack this capability. Undocking kinetics does not seem to be accelerated by the junction dynamics.

These results suggest that even small RNA enzymes can have highly complex structural dynamics, featuring a rugged energy landscape in the conformational space. Unlike in the case of large, multidomain RNA molecules, the level of complexity in the structural dynamics of small ribozymes had not been observed previously in ensemble experiments. These single-molecule studies make us wonder whether rugged energy landscapes and complex structural dynamics might very well be general properties of all RNAs, not just reserved for the large, complex RNA enzymes.

Single-Molecule Φ -Value Analysis

Characterization of the transition state ensemble is critical for a mechanistic understanding of the macromolecule folding problem. While transition-state analysis has been performed routinely for protein folding, its implementation in RNA folding has been challenging owing to the rugged energy landscape of RNA, which often leads to the coexistence of distinct structural transitions. The power of single-molecule measurements to isolate individual transitions makes them ideally suited for transition-state analysis of RNA folding. A single-molecule Φ -value analysis based on FRET was developed to characterize the degree to which specific intramolecular interactions are formed in the transition states of RNA folding (4, 7), following the Φ -value analysis introduced by Fersht (16) for characterizing protein folding.

Using FRET to monitor the folding and unfolding transitions of the hairpin ribozyme, in conjunction with metal-ion titrations and tertiary-contact-destabilizing mutations, Bokinsky et al. (7) showed that the transition state is compact with the Mg^{2+} -mediated interactions between the two domains of the hairpin ribozyme formed at a strength similar to that in the folded state, whereas the native tertiary contacts are not substantially formed. The conformational entropy loss and the breaking of secondary structures may be the primary mechanisms limiting the folding rate of the ribozyme. The folding transition states of some proteins also feature native-like topology without substantial native tertiary contacts (29).

This single-molecule Φ -value analysis is not only applicable to an elementary folding reaction of a small RNA enzyme, it can also be used to characterize the transition state of a local folding step of a large RNA enzyme (4, 81). After extensive effort to disrupt every single tertiary contact between the P1 duplex and catalytic core of the *Tetrahymena* ribozyme, Bartley et al. (4) have shown that not one of these tertiary contacts is formed in the transition state of P1-docking, which is believed to be the last folding step of the ribozyme. Their results suggest that P1-docking is rate-limited by kinetic traps. This work demonstrated the potential of this single-molecule Φ -value analysis to characterize each individual folding step of a complex, multistep, multipath folding reaction.

SINGLE-MOLECULE FRET STUDIES OF RNA CATALYSIS

Single-molecule methods are particularly well suited for detecting transient intermediate states and thereby resolving the microscopic steps of a complex reaction. This is demonstrated in the following two examples, which are at the two extremes of the ribozyme world, a small RNA enzyme and the ribosome.

Catalysis by a Small RNA Enzyme

Following the reaction of single hairpin ribozyme molecules using FRET, Zhuang et al. (82) have shown that this ribozyme catalyzes the cleavage reaction of its substrate in several distinct steps (Figure 2c): (a) substrate binding; (b) folding of the ribozyme-substrate complex into a catalytically active state (docking); (c) cleavage of the substrate; (d) the unfolding (undocking) of the ribozyme-product complex; and (e) release of the products. These results confirmed a previously proposed reaction pathway based on ensemble measurements (15, 72, 73). The determination of the rate constants of each step indicates that the overall cleavage reaction of the two-way junction ribozyme is primarily rate-limited by the two structural transition steps, (b) and (d), and by the internal equilibrium constants of the reversible cleavage step, (c) (82). The heterogeneous undocking kinetics mentioned above result in heterogeneous reaction kinetics.

The rate-limiting mechanism of the four-way junction ribozyme is different. Cleavage appears to dramatically accelerate undocking in the case of the four-way junction ribozyme. As a result the undocking rate is faster than the ligation rate, and the overall cleavage reaction rate is primarily limited by the internal cleavage reaction (35). In contrast, the undocking rate constant of the two-way junction ribozyme bound to its natural cleavage products is much slower than the ligation rate constant (51; G. Bokinsky, N. Walter & X. Zhuang, unpublished data). This discrepancy between the two-way and four-way ribozymes indicates an interesting allosteric effect that the junction has on the docked structure of the loop domains.

Translation by the Ribosome

Early works by Hochstrasser and coworkers (26, 65) have shown that surface-immobilized ribosome complexes retain their peptidyl transferase activity and can be detected at the single-complex level. These experiments gave researchers hope that single-molecule experiments might someday provide exciting new insights into the molecular mechanisms of protein synthesis in cells.

This promise has been realized in a recent, tour de force experiment by Blanchard et al. (5, 6). To set the stage for new discoveries, these authors first demonstrated that surface-immobilized ribosomes are highly active in tRNA accommodation and translocation and in peptide-bond formation (6). This is non-trivial considering the complexity of this vast ribonucleoprotein. Indeed specific surface immobilization and extensive surface passivation are necessary.

Next, using single-molecule FRET to follow the accommodation of the tRNA-elongation factor Tu-GTP ternary complex in real time, Blanchard et al. (5) resolved a multistep movement of the tRNA into the ribosome and identified the intermediate states in the tRNA-delivery pathway using antibiotics and nonhydrolyzable GTP analogs. Previously unknown intermediate states were discovered both in the initial codon-recognition steps and in the kinetic proofreading steps following GTP hydrolysis (Figure 3, see color insert). These results, complementing previous kinetic characterizations of translation (18, 49), have provided new insights into the structural basis for the initial cognate tRNA selection, kinetic proofreading, and thus for the fidelity of translation by the ribosome. Interesting dynamic tRNA fluctuations between the classical state and hybrid states have also been observed (6). The translocation of tRNAs from the classical to the hybrid state after peptide-bond formation has been suggested before; however, it is surprising that tRNA fluctuates spontaneously between these two states both before and after peptide-bond formation. This observation again highlights the capability of single-molecule experiments to resolve dynamic behaviors.

SINGLE-MOLECULE FORCE STUDIES OF RNA FOLDING AND UNFOLDING

Folding and Unfolding of Simple Secondary or Tertiary Structures

The use of mechanical forces to induce folding and unfolding provides a few unique advantages in characterizing macromolecule folding: (a) It allows the folding and unfolding reaction to occur along a well-defined reaction coordinate, the molecular end-to-end distance; and (b) the folding free energy can be readily measured (68).

Liphardt et al. (31) first characterized unfolding and refolding of small RNA molecules induced by mechanical forces using optical tweezers. They have shown that, with a slow-enough force-loading rate, the folding and unfolding of secondary structures, such as an RNA hairpin or a three-helix junction, can occur in equilibrium. In contrast, for a molecule with tertiary contacts, such as the P5abc domain of the *Tetrahymena* ribozyme in the presence of Mg^{2+} , folding and unfolding are nonequilibrium processes even at the slowest accessible force-loading rate. Interestingly, the transition state for folding for secondary structure formation is dramatically different from that for tertiary structure formation. In the former case, the transition state is relatively far from the folded state on the reaction coordinate, i.e., the molecular end-to-end distance, indicating a relatively soft transition. In contrast the transition state for tertiary structure folding is close to the folded state, suggesting that tertiary structures are much more brittle than secondary structures.

As promised, the free energy difference between the folded and unfolded states is readily calculated from the force-extension curves of molecules being folded or unfolded at equilibrium, and compare well with theoretical predictions.

Remarkably, the folding free energy can even be derived from force-extension curves of nonequilibrium (un)folding processes using a recently developed nonequilibrium statistical mechanics theorem, the Jarzynski's equality (30).

Unfolding of a Large, Multidomain RNA Enzymes

More recently, Onoa et al. (40) extended mechanical unfolding to a large RNA enzyme, the *Tetrahymena* ribozyme (Figure 4a, see color insert). The force-extension curves of individual RNA molecules show several discrete unfolding steps (Figure 4b), indicating the existence of multiple unfolding intermediate states. The molecular interactions disrupted at each of these unfolding steps have been identified by studying progressively larger pieces of the *Tetrahymena* ribozyme, determining the number of nucleotides released at each step, and using mutations or antisense oligonucleotides to perturb specific interactions in the RNA. Interestingly, most of these unfolding barriers are imposed by tertiary interactions, even though the overall thermodynamic stability of the molecule largely arises from the secondary structures, namely the base-paired helices. Unlike thermal or solution unfolding, in which tertiary structures are dissolved before secondary structures, the partially unfolded intermediates observed in this mechanical unfolding experiment contain both secondary structures and tertiary contacts.

Mechanical unfolding experiments have been used to characterize the secondary structure formation of RNA as large as the 16S ribosome RNA. Even in this case, well-defined unfolding intermediates are observed (23).

CONCLUSIONS AND FUTURE DIRECTIONS

A distinct advantage of single-molecule techniques is their capability to detect transient, nonaccumulative states and heterogeneous behavior. Such capabilities make these techniques well suited to investigate the structural dynamics and catalytic reactions of RNA molecules. Indeed, single-molecule experiments have already yielded important insights into RNA folding by detecting new folding intermediate states and pathways, and by revealing a daunting level of complexity in the conformational dynamics of both large and small RNA. Single-molecule experiments have also allowed us to better dissect the complex catalytic reactions of RNA enzymes by resolving individual reaction steps and discovering new reaction intermediates that are critical for understanding the reaction mechanisms.

What we have witnessed in the past few years is likely just the tip of the iceberg. Future single-molecule studies will certainly make more significant contributions to RNA science. As a growing number of essential reactions in cells have now been catalyzed by ribonucleoprotein enzymes, a bright future direction for single-molecule studies would be to characterize the folding, assembly, and enzymatic reactions of these important cellular ribonucleoproteins. The recent work on the

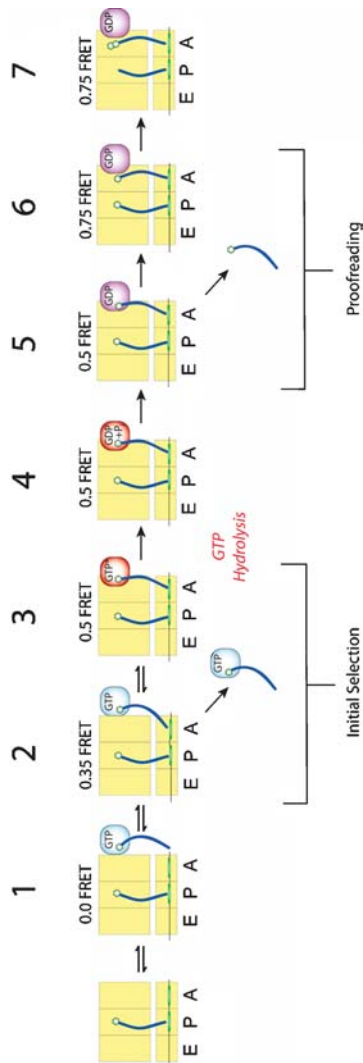


Figure 3 A model of tRNA accommodation into the A site of the ribosome inferred from a recent single-molecule FRET experiment and previous kinetic characterizations. The ribosome is shown in yellow. Exit (E), peptidyl (P), and aminoacyl (A) sites are depicted as yellow rectangles. tRNAs are shown in blue. The elongation factor Tu (EF-Tu) is shown in three colors (light blue, red, and purple), each corresponding to a different state. Step 1: Initial binding of the EF-Tu(GTP)tRNA ternary complex with the ribosome. Step 2: Contact is made between the anticodon of tRNA and the messenger RNA codon. Step 3: Productive codon recognition triggers the tRNA to move closer to the P site. Step 4: GTP hydrolysis. Step 5: EF-Tu changes from the GTP- to the GDP-bound form. Step 6: Release of tRNA from EF-Tu to accommodate at the peptidyl transferase center. Step 7: Peptide-bond formation. The value of FRET between the two tRNAs is indicated. Figure adopted from Reference 5.

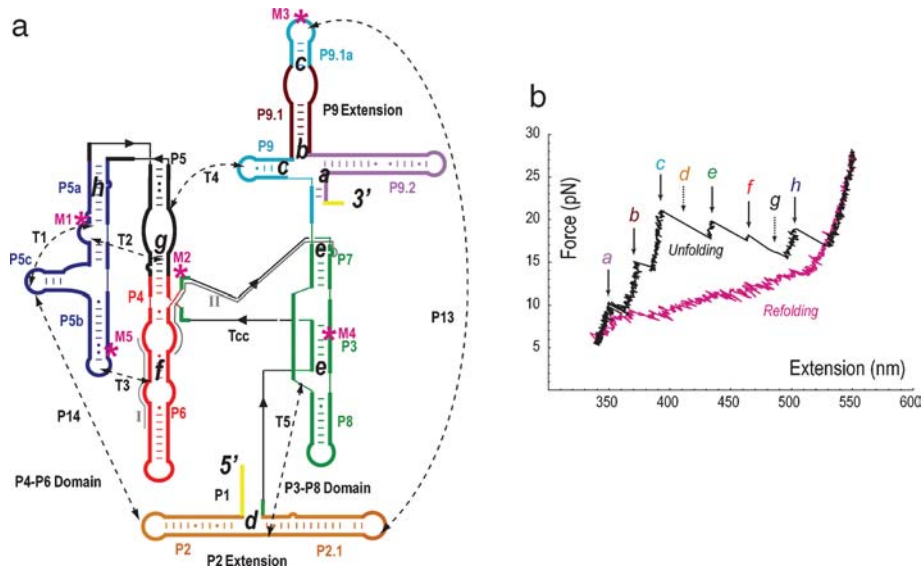


Figure 4 A single-molecule force study of the *Tetrahymena* ribozyme. (a) Secondary structure of the *Tetrahymena* ribozyme. Gray lines label sequences targeted by complementary DNA oligonucleotides, and “M” labels are site-directed mutations, both of which were used for identifying the molecular interactions disrupted at each unfolding step (kinetic barriers for mechanical unfolding). The letters *a* through *h* indicate the proposed positions of the kinetic barriers on the ribozyme. (b) Representative unfolding (*black*) and refolding (*pink*) force-extension curves of the ribozyme displaying several unfolding steps. The letters *a* through *h* correspond to the kinetic barriers. Figure adopted from Reference 40.