Single-Molecule RNA Folding

GREGORY BOKINSKY† AND
XIAOWEI ZHUANG*,†,‡

Department of Chemistry and Chemical Biology and Department of Physics, Harvard University, Cambridge, Massachusetts 02138

Received July 19, 2004

ABSTRACT

Single-molecule experiments significantly expand our capability to characterize complex dynamics of biological processes. This relatively new approach has contributed significantly to our understanding of the RNA folding problem. Recent single-molecule experiments, together with structural and biochemical characterizations of RNA at the ensemble level, show that RNA molecules typically fold across a highly rugged energy landscape. As a result, long-lived folding intermediates, multiple folding pathways, and heterogeneous conformational dynamics are commonly found for RNA enzymes. While initial results have suggested that stable secondary structures are partly responsible for the rugged energy landscape of RNA, a complete mechanistic understanding of the complex folding behavior has not yet been obtained. A combination of single-molecule experiments, which are well suited to analyze transient and heterogeneous dynamic behaviors, with ensemble characterizations that can provide structural information at a superior resolution will likely provide more answers.

1. Introduction

Since the groundbreaking discovery that certain RNA molecules are catalysts,[1,2] a growing list of cellular reactions are seen to be catalyzed by 'ribozymes' or RNA enzymes. Important functions such as translation, pre-messenger RNA splicing, and tRNA maturation are carried out by ribonucleoproteins that use the RNA constituents to perform major catalytic functions.[2−6] In addition, RNA demonstrates extensive versatility in modern biotechnology and medicine, as a gene regulatory tool,[7] as a therapeutic agent,[8] and for high-throughput drug discovery.[9] Our growing appreciation of RNA's considerable importance has not yet been matched by a thorough understanding of how linear RNA sequences fold into specific structures that mediate complex functions. However, even at this early stage RNA folding has already proven to be as rich and fascinating as its protein folding counterpart.[10−31]

A distinct property of RNA folding is its hierarchical nature.[32] RNA molecules fold by forming secondary structures (local duplexes and single-stranded loops) before arranging tertiary structures (interactions between the secondary structural elements). As a result of the relatively stable secondary structures, either non-native or native but formed at the wrong time, RNA is easily caught in misfolded configurations (kinetic traps) that must be resolved before the native structure can be attained.[17,22,25] Additional folding traps also exist due to non-native tertiary structures or native ones that formed too early.[15,18,33]

RNA enzymes often follow many pathways and traverse multiple intermediate states before attaining native structures.[10−31] For example, probing the RNA structure during the folding process using oligonucleotide hybridization, hydroxyl radical foot printing, circular dichroism, or UV absorption assays has led to the discovery that RNA folding occurs through a series of intermediate states.[10−12,14,15] The hydroxyl radical foot-printing assay is particularly impressive in that it offers structural information at nucleotide resolution with millisecond time resolution.[14] RNA enzymes were seen to fold along multiple pathways by native gel electrophoresis and enzymatic activity assays.[13,17,19,22] However, these methods that measure the average property of an ensemble of molecules can only probe accumulative intermediate states and significantly populated pathways but cannot detect non-accumulative, 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 due to their intrinsic capability to detect nonaccumulative transient intermediate states and heterogeneity in the system. Here, we review recent developments in the field of RNA folding that have been made possible by single-molecule experiments, focusing on those using fluorescence resonance energy transfer (FRET).[21,24,25,28−31,34−37] Single-molecule force studies of RNA folding/unfolding[38−40] will not be covered in this review.

2. Single-Molecule FRET

FRET is a powerful technique for studying the structural dynamics of biomolecules.[41−43] In this method fluorescent donor and acceptor dyes are attached to two specific sites of a molecule. The induced 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

$$E = 1/(1 + (R/R_0)^6) \quad (1)$$

where R is the distance between the two dyes. R_0, the Förster radius, is typically 3−8 nm, making FRET sensitive to changes of a few nanometers in the distance between

Gregory Bokinsky received his B.A. degree in Biochemistry from Swarthmore College. He is a Ph.D. candidate in the Department of Chemistry and Chemical Biology at Harvard University.

Xiaowei Zhuang received her undergraduate education in Physics from the University of Science and Technology of China and her doctoral training in Physics from the University of California at Berkeley. After a postdoctoral study at Stanford University she joined the Faculty of Harvard University in 2001, holding a joint appointment in the Department of Chemistry and Chemical Biology and the Department of Physics. Her research interests lie in the fields of single-molecule biophysics and cellular imaging. Her current research focuses on viral entry mechanisms and protein−nucleic acid interactions.

* To whom correspondence should be addressed. Phone: 617-496-9558. Fax: 617-496-9559. E-mail: zhuang@chemistry.harvard.edu.
† Department of Chemistry and Chemical Biology.
‡ Department of Physics.

10.1021/ar040142o CCC: $30.25 © 2005 American Chemical Society
Published on Web 02/24/2005

the donor and acceptor. The energy-transfer efficiency also depends on the orientation of the two dyes. This introduces uncertainty into FRET-based distance determination, a problem that 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, we have found that the best way to correlate a FRET change to a specific change in conformation is to introduce mutations that specifically perturb the conformational change.[21,24]

The development of single-molecule fluorescence spectroscopy allows FRET to be measured at the single-molecule level.[44,45] Using an RNA three-way junction as model system it was shown that ligand-induced conformational changes of an RNA could be monitored by FRET at the single-molecule level.[46] RNA conformational dynamics over a wide range of time scales have been probed by single-molecule FRET, ranging from milliseconds to minutes or even hours.[21,24,30] This range was further extended into the microsecond range for a reversible conformational transition using fluorescence correlation spectroscopy.[37] Here, we focus on the exploration of RNA folding by single-molecule FRET.

3. Folding and Structural Dynamics of Small Ribozymes

Much of our knowledge of RNA structure has been obtained from the study of small RNA molecules. The structures of small ribozymes have provide detailed characterization of RNA tertiary contact motifs as well as insights into how ribozymes catalyze reactions.[47−51] However, these largely static structures provide little information about how small RNAs fold. Recent single-molecule studies have filled this gap by revealing the rich, dynamic nature of small ribozymes.[24,29,30,34−37]

A small ribozyme that has been studied extensively by single-molecule FRET is the hairpin ribozyme, one of the best-characterized small ribozymes.[52,53] The structure of the hairpin ribozyme was revealed in two recent high-resolution crystal structures.[50,51] Two forms of hairpin ribozymes have been studied at the single-molecule level. Those are the two-way junction ribozyme, a minimal hairpin ribozyme with high enzymatic activity, and the wild-type ribozyme that has a four-way junction.[52,53]

To observe the structural dynamics of single hairpin ribozyme molecules we attached a fluorescent donor and an acceptor dye to the ends of the helix−loop−helix domains (Figure 1A).[24] FRET trajectories of individual molecules of the two-way junction ribozyme have allowed us to directly observe the undocked, docked, and substrate-free states of the ribozyme (Figure 1B) and thus to dissect the entire cleavage pathway into five distinct steps (Figure 1C): (i) substrate binds to the ribozyme, forming the extended, catalytically inactive, undocked state; (ii) loop domains A and B dock, forming the catalytically active state; (iii) the ribozyme cleaves the substrate; (iv) the ribozyme−product complex undocks; (v) the products are released. These results confirmed previously proposed reaction pathway-based ensemble measurements.[52−54]

Single-molecule FRET experiments have demonstrated a highly complex folding dynamics for this small ribozyme.[24] While previous ensemble FRET experiments have shown the existence of docked and undocked conformations,[54,55] single-molecule experiments have allowed the reversible docking and undocking transitions of individual molecules to be observed in real time and the docking and undocking kinetics to be quantitatively determined (Figure 2A). The two-way junction ribozyme docks with a single rate constant but undocks with at least four different rate constants.[24] Furthermore, the molecules exhibit a memory effect: ribozymes retain their particular undocking rates through many docking−undocking cycles, and conversion between different undocking behaviors requires at least several hours.[24] This suggests the existence of at least four different docked states and four different undocked states, which is remarkable for such a small RNA enzyme. This type of heterogeneous docking/undocking kinetics was not unique to the wild-type two-way junction ribozyme but was generally observed for ribozymes with modifications that significantly affect the docking and undocking rate constants.[34] A study on the four-way junction hairpin ribozyme also shows pronounced heterogeneity in docking−undocking kinetics.[30] A distinct property of the four-way junction ribozyme is that docking occurs 2−3 orders of magnitude faster.[30] This dramatic increase is a result of the intrinsic structural dynamics of the four-way junction: the ribozyme appears to dock via an intermediate state in which the four-way junction juxtaposes the loop domains into proximity but without substantial loop−loop interactions.[30] This result is further supported by a single-molecule study of hairpin ribozymes freely diffusing in solution.[36] Even when the two loops are replaced with perfectly base-paired helices, these helices are still juxtaposed into proximity by the four-way junction, while both two-way and three-way junctions lack this capability.[36] The undocking kinetics does not seem to be accelerated by the junction dynamics.

One might wonder whether the heterogeneity observed in these studies is an experimental artifact introduced by modifications of RNA molecules that facilitates single-molecule experiments. However, the enzymatic activity of hairpin ribozymes determined from single-molecule experiments is quantitatively similar to results from ensemble experiments.[24] Moreover, the heterogeneous structural dynamics seen in single-molecule experiments is quantitatively consistent with the heterogeneous cleavage kinetics observed in ensemble experiments.[24,34] Similar heterogeneity was also detected in liposome-encapsulated hairpin ribozymes, which are most likely free from surface perturbations.[56] Heterogeneity in conformational dynamics and reaction kinetics was also observed in large RNA enzymes,[31] ribonucleoproteins (our unpublished results), and protein enzymes.[57,58] These observations suggest that the observed heterogeneous structural dynamics, rather than being an experimental artifact, may be a general property for RNA and proteins, which has gone under-

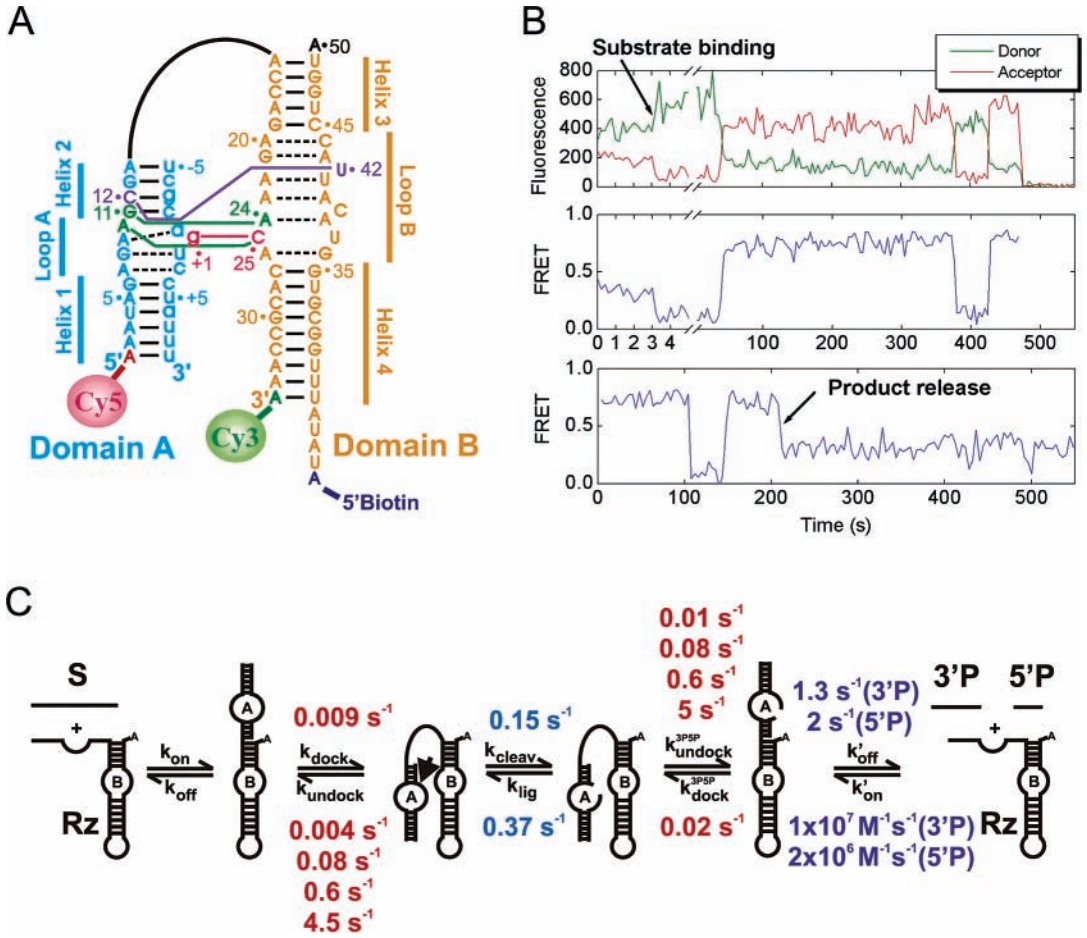

FIGURE 1. Reaction pathway of the hairpin ribozyme. (A) The two-way junction form of the ribozyme and a FRET labeling scheme. The Cy3 and Cy5 dyes serve as the fluorescent donor and acceptor. The ribozyme was immobilized to the surface via a biotin−streptavidin interaction. Tertiary interactions are indicated by the following colors: red, g+1:C25 Watson−Crick base pair; green, ribose zipper; purple, U42 binding pocket. (B) Following the structural dynamics and function of single ribozyme molecules. (Top and middle) A fluorescence time trace of a single ribozyme that shows binding of a noncleavable substrate, docking, and undocking. Green and red lines are the fluorescence signals from the donor and acceptor, respectively. The blue line is the FRET value, defined as the acceptor signal divided by the sum of the donor and acceptor signals. The substrate-free ribozyme gives a FRET value at 0.4. Upon binding of a substrate the FRET value changes to 0.2, indicating an undocked state. The FRET then exhibits stochastic transitions between two levels (0.2 and 0.8) that correspond to the undocked and docked states, respectively. (Bottom) FRET time trace of a single complex of the ribozyme with a cleavable substrate showing docking, undocking, and cleavage. The release of products after cleavage recovers the ribozyme to a FRET value of 0.4. (C) The reaction pathway of the hairpin ribozyme. Rz, S, and 3′P and 5′P stand for the ribozyme, the substrate, and the 3′ and 5′ cleavage products, respectively. The rate constants were obtained from single-molecule FRET experiments in conjunction with ensemble cleavage and ligation assays.[24,34]

appreciated so far due to the lack of effective experimental tools. Thus far, the structural basis of the heterogeneity remains quite elusive.

The docking transitions of the two-way junction hairpin ribozyme are surprisingly slow. To determine the mechanism underlying the slow docking transition we characterized the docking transition state using Φ-value analysis.[29] A Φ value reflects the degree to which a particular tertiary contact is formed in the transition state and is calculated by

$$\Phi = \Delta\Delta G^{\ddagger}_{\mathrm{dock}}/(\Delta\Delta G^{\ddagger}_{\mathrm{dock}} - \Delta\Delta G^{\ddagger}_{\mathrm{undock}}) \qquad (2)$$

where $\Delta\Delta G^{\ddagger}_{(\mathrm{un})\mathrm{dock}}$ is the mutation-induced change in the free energy barrier for (un)docking.[59] Using site-specific mutations to destabilize each of the three sets of tertiary interactions existing in the docked state (Figure 1A) we found that the tertiary contacts that stabilize the docked structure are not substantially formed in the transition state (Figure 3A and B). The metal-ion titration experiments suggest that the two loop domains of the ribozyme are in close proximity in the transition state. This compact transition state (ensemble) has lower conformational entropy than the extended configurations and may thus present a bottleneck in the docking transition. This notion of the entropic barrier is consistent with the faster docking behavior observed for the four-way junction ribozyme in which the stacking interactions in the four-way junction counterbalance the entropic cost and help to bring the two loop domains into close proximity. The entropic barrier is probably not the only reason the docking transition is slow. Stacking interaction between helices in the undocked state also contributes partially to the docking barrier.[29] The disruption of RNA secondary struc-

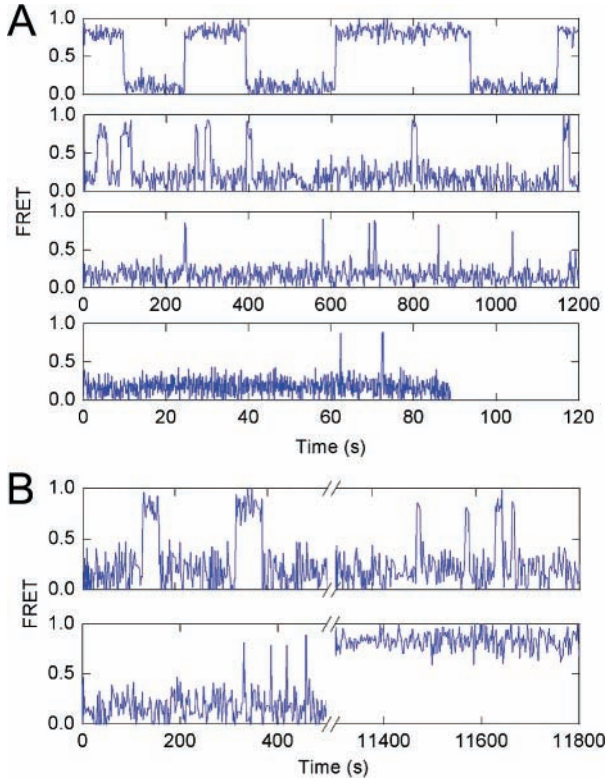

FIGURE 2. Heterogeneous conformational dynamics of the hairpin ribozyme. (A) FRET time traces of individual ribozyme molecules show heterogeneous undocking kinetics with dramatically different rate constants. The distributions of dwell times of undocked and docked states, obtained from hundreds of time traces, indicate that the wild-type two-way junction ribozyme docks with a single rate constant but undocks with at least four different rate constants given in Figure 1C.[24,34] Each rate constant is exemplified by one FRET trace. The molecules retain memory of their undocking kinetics for many hours. (B) Examples of memory loss after 3 h. The upper(/lower) trace indicates an increase(/decrease) in the undocking rate constant after 3 h.

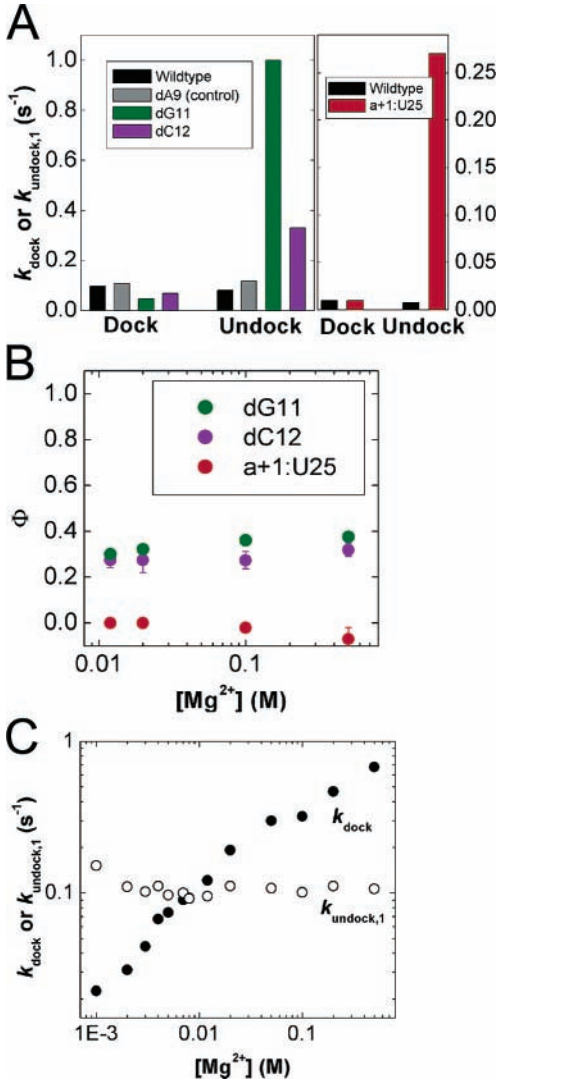

FIGURE 3. Transition-state analysis of docking of the hairpin ribozyme. (A) Effect of mutations on the rate constants for docking and undocking. The columns show the rate constant for docking (k_{dock}) and the slowest rate constants for undocking ($k_{undock,1}$) of the wild-type and various mutant two-way junction ribozymes at 12 mM Mg^{2+} and 37 °C (left) and at 12 mM Mg^{2+} and 25 °C (right). The dG11 mutation destabilizes the ribose zipper motif. The dC12 mutation destabilizes the U42 binding pocket. The a+1:U25 mutation replaces the g+1:C25 with a weaker Watson–Crick base pair. (B) Φ values of the above modifications at various Mg^{2+} concentrations. The low Φ values indicate that the corresponding tertiary interactions are not formed to a substantial extent in the transition state(s). (C) Dependence of k_{dock} and $k_{undock,1}$ of the wild-type two-way junction ribozyme on Mg^{2+} concentration at 37 °C.[29]

ture within the loop domains provides another obstacle to docking.[50,60,61] Such entropic costs and secondary structure corrections are probably two general reasons why structural transitions in RNA molecules are slow.

The slow and heterogeneous docking/undocking kinetics reflect a rugged folding energy landscape. Such a rugged landscape not only dictates folding dynamics but also influences the enzymatic activity of the ribozyme. A combination of single-molecule FRET, ensemble assays, and kinetic simulations suggests that the overall cleavage kinetics of the two-way junction ribozyme is strongly limited by structural transition steps (docking and undocking) and the internal equilibrium constants of the reversible cleavage reaction (Figure 1C).[24,34] The heterogeneous conformational dynamics result in heterogeneous reaction kinetics.[24,34] The rate-limiting mechanism of the four-way junction ribozyme is, however, different. It appears that cleavage dramatically accelerates undocking in the case of the four-way junction ribozyme. As a result, the undocking rate constant is faster than that of ligation and the overall cleavage reaction rate is primarily limited by the internal cleavage reaction.[35] By contrast, the undocking rate constant of the two-way junction ribozyme complexed with its natural cleavage products is much slower than the ligation rate constant[34] (Bokinsky, G.; Walter, N.; Zhuang, X. Unpublished results). This discrepancy between the two-way and four-way junction ribozymes indicates an interesting allostery effect that the junction has on the docked structure of the loop domains.

Perhaps the most surprising finding from these single-molecule studies is that even a small RNA molecule, such as the hairpin ribozyme, shows very complex, heteroge-

neous structural dynamics.[24,30,34] This discovery would have been difficult to make without single-molecule experiments. In addition, the above results also clearly demonstrate the power of single-molecule measurements to dissect a complex, multistep reaction and determine the rate constant of each individual reaction step.[24,34,35]

4. Folding Dynamics of Large, Multidomain Ribozymes

Large ribozymes typically exhibit more complex folding dynamics than small RNAs due to the many stable interactions that can form between different domains and the intricate interplay between these interactions. Recent single-molecule studies on large, multidomain RNAs[21,25,28,31] have improved our understanding of RNA folding, complementing results from ensemble characterizations using various foot-printing methods, enzymatic activity assays, and optical scattering or spectroscopic techniques.[10−20,22,23,25−27]

The first single-molecule RNA folding study used the *Tetrahymena* ribozyme, one of the best-studied large ribozymes, as a model system.[21] The power of single-molecule FRET is apparent in this early work. Dynamic properties that are difficult to analyze with ensemble experiments, including nonaccumulative intermediate states, parallel folding pathways, and equilibrium conformational fluctuations, are directly observed in single-molecule FRET trajectories.

At equilibrium the 5′-end duplex (P1) of the *Tetrahymena* ribozyme spontaneously docks with and undocks from the active core of the folded ribozyme, as vividly demonstrated in FRET time traces of individual molecules (Figure 4A and B). A single-molecule Φ-value analysis has shown that none of the tertiary contacts are formed in the transition state of P1 docking,[28] similar to the hairpin ribozyme. However, in contrast to the strong Mg^{2+}-dependent docking found for the hairpin ribozyme, the P1 duplex docking exhibits little Mg^{2+} dependence. The rate-limiting mechanism was suggested to be due to a kinetic trap(s), instead of conformational searching, based on the observation that a modification that increases the degree of freedom between the P1 duplex and the ribozyme core accelerates docking. Considering the capability of single-molecule experiments to isolate an individual transition step in a complex, multistep process, single-molecule Φ-value analysis can, in principle, be used to characterize the transition state of any reaction step of a multistep reaction.

Single-molecule FRET trajectories recorded during the folding course of ribozyme directly reveal kinetic folding intermediates.[21] 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) (Figure 5A). The increase to 0.3 coincides with a rapid, electrostatic collapse of the ribozyme before substantial formation of tertiary structure.[26] The increase of the FRET to 0.9 coincides with attainment of the native structure.[21] A number of folding intermediates identified

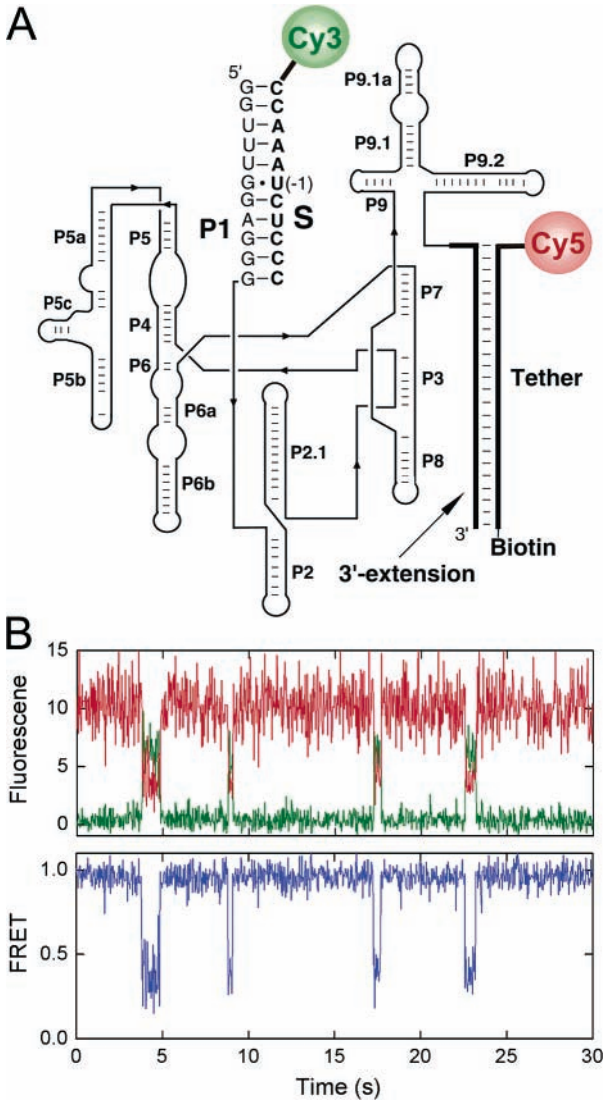

FIGURE 4. Single-molecule FRET study of *Tetrahymena* ribozyme. (A) Labeling scheme of the *Tetrahymena* ribozyme. The Cy3 and Cy5 dyes serve as the fluorescent donor and acceptor. The ribozyme was immobilized to the surface via a biotin−streptavidin interaction. (B) Time trajectories showing the docking and undocking of the P1 duplex of a single ribozyme molecule at equilibrium. Red trace: the donor signal. Green trace: the acceptor signal. Blue trace: the FRET value. The FRET levels of 0.3 and 0.9 correspond to the undocked and docked states of P1, respectively.

in previous studies[10,14,15] were not distinguished in this study, presumably because the dye labeling scheme used yields the same FRET value for these states. It is thus necessary to vary the attachment sites of donors and acceptors to allow a more thorough characterization of the folding dynamics. This is especially important for multidomain RNAs that have a large number of tertiary interactions.

Nevertheless, this initial study has already illustrated a complex network of folding pathways taken by the *Tetrahymena* ribozyme.[21] A histogram of folding times obtained from single-molecule FRET trajectories yields two distinct folding rates, 1 s^{-1} and 1 min^{-1} (Figure 5B). In addition, a large fraction of molecules was trapped in

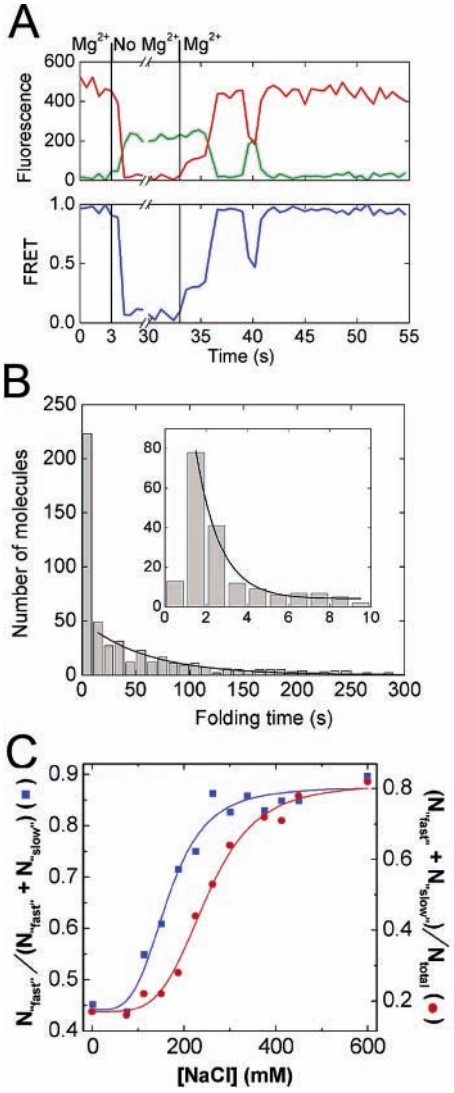

FIGURE 5. Complex folding pathways of the *Tetrahymena* ribozyme. (A) Folding time trajectories showing the unfolding and folding of a single ribozyme molecule. Red trace: the donor signal. Green trace: the acceptor signal. Blue trace: the FRET value. The unfolding and refolding were triggered by removing and adding Mg^{2+} at 3 and 30 s, respectively. The transient decrease at ~40 s is a P1-duplex undocking event. (B) Folding time histogram. The line is a single-exponential fit with the first bin excluded, giving a rate constant of 1 min^{-1}. (Inset) Expansion of the first bin of the main histogram. Relatively few molecules fold in the first second due to a delay between the initiation of refolding buffer and its arrival at the sample. The line is a single-exponential fit giving a rate constant of 1 s^{-1}. (C) Partition of the molecules into the "fast", "slow", and "misfolding" pathways at various initial Na^+ concentrations. $N_{\text{"fast"}}$ and $N_{\text{"slow"}}$ stand for the numbers of molecules folded with 1 s^{-1} and 1 min^{-1} rate constants. $N_{\text{"total"}}$ stands for the total number of molecules.

an extremely long-lived, misfolded state and eventually folded to the native state after many hours. Altogether, theses results indicate that the *Tetrahymena* ribozyme folds along at least three distinct pathways, designated the "fast", "slow", and "misfolding" pathways. While the latter two pathways have been observed previously in ensemble measurements,[13,17,19,22] the most rapid pathway was first observed in the single-molecule FRET experiment. The existence of multiple pathways indicates that the RNA folds across a highly rugged energy landscape.

Preincubation of the ribozyme with different concentrations of monovalent salt dramatically alters the partition among the "fast", "slow", and "misfolding" pathways (Figure 5C).[25] The cause of this redistribution was originally attributed to changes in the unfolded (secondary) structure induced by monovalent salt based on the following three observations.[25] (1) Monovalent salt promotes specific secondary structures. In particular, formation of the P13 or P3 duplex exhibits a similar Na^+ concentration dependence, respectively, to that of the repartitioning behavior from the "slow" to "fast" or from the "misfolding" to correctly folding pathways. (2) P13 stabilizes the folded structure of the ribozyme and influences folding kinetics.[25,62] A modification that stabilizes P3 prevents the ribozymes from misfolding.[17,25] (3) The ribozyme in monovalent salt is significantly less compact compared to the compaction intermediates observed on the folding pathway, which precede the first intermediate state with tertiary interactions. However, recent results from a hydroxyl radical foot-printing assay suggest that high concentrations of monovalent salt induce an extensive amount of tertiary structures in addition to secondary structure changes.[27] Thus, the changes in the folding kinetics induced by preincubation with monovalent salt may not be solely caused by changes in the secondary structures but by preformed tertiary structures as well.

The rugged energy landscape of RNA is also demonstrated in a single-molecule FRET study of another large RNA molecule, the catalytic domain of *Bacillus subtilis* RNase P RNA.[31] Ensemble characterizations of this enzyme indicate the existence of three thermodynamically stable conformational states: the unfolded state (U), the intermediate state (I_{eq}), and the native state (N). However, analysis of the molecules at equilibrium using single-molecule FRET suggests at least seven different thermodynamically stable conformations, as distinguished by their corresponding FRET levels and connectivity between different states. While at low or high Mg^{2+} concentrations (unfolding or folding conditions, respectively) the molecules exhibit a constantly low or high FRET level, at intermediate concentrations the molecules exhibit very complex dynamics. In contrast to the two- or three-state transitions observed in the equilibrium FRET traces of the Tetrahymena or hairpin ribozyme, the FRET time traces of the RNase P RNA often exhibit broad, continuous fluctuations over a wide range of FRET values. The transition behavior shows a high degree of heterogeneity from molecule to molecule. This result suggests that the molecules are broadly scattered among distinct regions of a rugged folding landscape, each with its own characteristic set of metastable states. It is interesting to ask whether such complex equilibrium dynamics also occurs in other large RNA molecules.

5. Summary and Future Directions

A distinct advantage of single-molecule techniques is their capability to detect transient, nonaccumulative states and heterogeneous behavior. Such capability makes these techniques well suited to investigate folding mechanisms of biomolecules. When applied to RNA folding, single-molecule experiments show that RNA molecules exhibit highly complex folding and equilibrium dynamics. Even a small RNA like the hairpin ribozyme can adopt many different conformational states separated by large energetic barriers at equilibrium. The folding dynamics of a large, multidomain RNA is even more sophisticated, consistent with results from ensemble experiments. A general picture of RNA folding begins to form: RNA molecules tend to have rugged energy landscapes, much more so than proteins of similar sizes. As a result, multiple folding pathways, long-lived folding intermediates, and heterogeneous equilibrium dynamics appear to be ubiquitous. The fundamental mechanism underlying this ruggedness is still not completely clear, although the stable secondary structures of RNA definitely play an important role.

The complex and slow structural dynamics resulting from rugged landscapes can impose severe limits on the catalytic capabilities of RNA enzymes. As some of the most essential cellular reactions are performed by RNA enzymes, a critical question arises: how does nature overcome the limits imposed by the frustrated structural dynamics of RNA? The solution lies partly in protein cofactors. Roles of protein cofactors in stabilizing the functional architecture of RNA, chaperoning the folding reaction, and promoting ligand binding and dissociation have been indicated.[63−65] Exploration of the folding dynamics of ribonucleoproteins is still in its infancy and presents an exciting future direction for single-molecule studies. It would also be extremely interesting to monitor the folding behavior of individual RNA molecules in vivo, although many technical difficulties have to be overcome before this can be realized.

Finally, we comment on the shortcomings of single-molecule techniques. The small amount of signal that can be collected from a single molecule fundamentally limits the structural resolution that such techniques can achieve. As a result, although single-molecule experiments can in principle detect transient states and heterogeneous behavior better than other approaches, it is often difficult to obtain a high-resolution structure of such transient states or the structural basis of the heterogeneity. This can be partly overcome by intelligent placement of the fluorescent donor and acceptor on the biomolecule or by mutations that specifically perturb the structural dynamics. Even so, the best structural resolution that single-molecule approaches can achieve probably will not match some of the ensemble techniques such as chemical footprinting methods. A full understanding of RNA folding can only come from integrated knowledge provided by these different experimental approaches together with theoretical modeling and simulation of real RNA molecules, which have become realistic due to recent technology developments.[66,67]

References

(1) Kruger, K.; Grabowski, P. J.; Zaug, A. J.; Sands, J.; Gottschling, D. E. et al. Self-splicing RNA−Auto-excision and auto-cyclization of the ribosomal-RNA intervening sequence of Tetrahymena. *Cell* **1982**, *31*, 147−157.

(2) Guerrier-Takada, C.; Gardinier, K.; Pace, T.; Altman, S. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* **1983**, *35*, 849−857.

(3) Noller, H. F.; Hoffarth, V.; Zimniak, L. Unusual resistance of peptidyl transferase to protein extraction procedure. *Science* **1992**, *265*, 1709−1712.

(4) Steitz, T. A.; Moore, P. B. RNA, the first macromolecular catalyst: the ribosome is a ribozyme. *Trends Biochem. Sci.* **2003**, *28*, 411−418.

(5) Valadkhan, S.; Manley, J. L. Intrinsic metal binding by a spliceosomal RNA. *Nat. Struct. Biol.* **2002**, *9*, 498−9.

(6) Villa, T.; Pleiss, J. A.; Guthrie, C. Spliceosomal snRNAs: Mg2+-dependent chemistry at the catalytic core? *Cell* **2002**, *109*, 149−152.

(7) Famulok, M.; Verma, S. In vivo-applied functional RNAs as tools in proteomics and genomics research. *Trends Biotechnol.* **2002**, *20*, 462−6.

(8) Sullenger, B. A.; Gilboa, E. Emerging clinical applications of RNA. *Nature* **2002**, *418*, 252−8.

(9) Burgstaller, P.; Jenne, A.; Blind, M. Aptamers and aptazymes: accelerating small molecule drug discovery. *Curr. Opin. Drug Discov. Devel.* **2002**, *5*, 690−700.

(10) Zarrinkar, P. P.; Williamson, J. R. Kinetic Intermediates in RNA Folding. *Science* **1994**, *265*, 918−924.

(11) Zarrinkar, P. P.; Wang, J.; Williamson, J. R. Slow folding kinetic of RNase P RNA. *RNA* **1996**, *2*, 564−573.

(12) Pan, T.; Sosnick, T. R. Intermediates and kinetics traps in the folding of a large ribozyme reveal by circular dichroism and Uv absorbance spectroscopies and catalytic activity. *Nat. Struct. Biol.* **1997**, *4*, 931−938.

(13) Pan, J.; Thirumalai, D.; Woodson, S. A. Folding of RNA involves parallel pathways. *J. Mol. Biol.* **1997**, *273*, 7−13.

(14) Sclavi, B.; Sullivan, M.; Chance, M. R.; Brenowitz, M.; Woodson, S. A. RNA folding at millisecond intervals by synchrotron hydroxyl radical footprinting. *Science* **1998**, *279*, 1940−1943.

(15) Treiber, D. K.; Rook, M. S.; Zarrinkar, P. P.; Williamson, J. R. Kinetic intermediates trapped by native interactions in RNA folding. *Science* **1998**, *279*, 1943−6.

(16) Rook, M. S.; Treiber, D. K.; Williamson, J. R. Fast folding mutants of the Tetrahymena group I ribozyme reveal a rugged folding energy landscape. *J. Mol. Biol.* **1998**, *281*, 609−20.

(17) Pan, J.; Woodson, S. Folding intermediates of a self-splicing RNA: Mis-pairing of the catalytic core. *J. Mol. Biol.* **1998**, *280*, 597−609.

(18) Pan, T.; Fang, X.; Sosnick, T. R. Pathway modulation, circular permutation and rapid folding under kinetic control. *J. Mol. Biol.* **1999**, *286*, 721−731.

(19) Russell, R.; Herschlag, D. New pathways in folding of the Tetrahymena group I RNA enzyme. *J. Mol. Biol.* **1999**, *291*, 1155−1167.

(20) Buchmueller, K. L.; Webb, A. E.; Richardson, D. A.; Weeks, K. M. A collapsed non-native RNA folding state. *Nat. Struct. Biol.* **2000**, *7*, 362−6.

(21) Zhuang, X.; Bartley, L.; Babcock, H.; Russell, R.; Ha, T. et al. A single molecule study of RNA catalysis and folding. *Science* **2000**, *288*, 2048−2051.

(22) Pan, J.; Deras, M. L.; Woodson, S. A. Fast folding of a ribozyme by stabilizing core interactions: Evidence for multiple folding pathways in RNA. *J. Mol. Biol.* **2000**, *296*, 133−144.

(23) Heilman-Miller, S. L.; Pan, J.; Thirumalai, D.; Woodson, S. A. Role of counterion condensation in folding of the Tetrahymena ribozyme II. Counterion-dependence of folding kinetics. *J. Mol. Biol.* **2001**, *309*, 57−68.

(24) Zhuang, X.; Kim, H.; Pereira, M.; Babcock, H.; Walter, N. et al. Correlating of Structural Dynamics and Function in Single Ribozyme Molecules. *Science* **2002**, *296*, 1473−1477.

(25) Russell, R.; Zhuang, X.; Babcock, H.; Millett, I. S.; Doniach, S. et al. Exploring the Folding Landscape of a Structured RNA. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 155−160.

(26) Russell, R.; Millett, I. S.; Tate, M. W.; Kwok, L. W.; Nakatani, B. et al. Rapid compaction during RNA folding. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4266−71.

(27) Takamoto, K.; He, Q.; Morris, S.; Chance, M. R.; Brenowitz, M. Monovalent cations mediate formation of native tertiary structures of the *Tetrahymena thermophila* ribozyme. *Nat. Struct. Biol.* **2002**, *9*, 928−933.
(28) Bartley, L. E.; Zhuang, X.; Das, R.; Chu, S.; Herschlag, D. Exploration of the transition state for tertiary structure formation between RNA helix and a large structured RNA. *J. Mol. Biol.* **2003**, *328*, 1011−1026.
(29) Bokinsky, G.; Rueda, D.; Misra, V. K.; Gordus, A.; Rhodes, M. M.; Babcock, H. P.; Walter, N. G.; Zhuang, X. Single-molecule transition-state analysis of RNA folding. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9302−9307.
(30) Tan, E.; Wilson, T. J.; Nahas, M. K.; Clegg, R. M.; Lilley, D. M. et al. A four-way junction accelerates hairpin ribozyme folding via a discrete intermediate. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9308−9313.
(31) Xie, Z.; Srividya, N.; Sosnick, T. R.; Pan, T.; Scherer, N. F. Single-molecule studies highlight conformational heterogeneity in the early folding steps of a large ribozyme. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 534−539.
(32) Brion, P.; Westhof, E. Hierarchy and dynamics of RNA folding. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 113−37.
(33) Treiber, D. K.; Williamson, J. R. Concerted kinetic folding of a multidomain ribozyme with a disrupted loop-receptor interaction. *J. Mol. Biol.* **2001**, *305*.
(34) Rueda, D.; Bokinsky, G.; Rhodes, M. M.; Rust, M. J.; Zhuang, X. et al. Single-molecule enzymology of RNA: Essential functional groups impact catalysis from a distance. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10066−10071.
(35) Nahas, M. K.; Wilson, T. J.; Hohng, S. C.; Jarvie, K.; Lilley, D. M. J. et al. Observation of internal cleavage and ligation reactions of a ribozyme. *Nat Struct. Mol. Biol* **2004**, *11*, 1107−1113.
(36) Pljevaljcic, G.; Millar, D. P.; Deniz, A. A. Freely diffusing single hairpin ribozymes provide insights into the role of secondary structure and partially folded states in RNA folding. *Biophys. J.* **2004**, *87*, 457−467.
(37) Kim, H. D.; Nienhaus, G. U.; Ha, T.; Orr, J. W.; Williamson, J. R. et al. Mg2+-dependent conformational change of RNA studied by fluorescence correlation and FRET on immobilized single molecules. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4284−4289.
(38) Liphardt, J.; Onoa, B.; Smith, S. B.; Tinoco, I.; Bustamante, C. Reversible unfolding of single RNA molecules by mechanical force. *Science* **2001**, *292*, 733−737.
(39) Onoa, B.; Dumont, S.; Liphardt, J.; Smith, S. B.; Tinoco, I. et al. Identifying kinetic barriers to mechanical unfolding of the T-thermophila ribozyme. *Science* **2003**, *299*, 1892−1895.
(40) Tinoco, I. Force as a useful variable in reaction: unfolding RNA. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 363−385.
(41) Stryer, L.; Haugland, R. P. Energy transfer: a spectroscopic ruler. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 719−726.
(42) Selvin, P. R. Fluorescence resonance energy-transfer. *Methods Enzymol.* **1995**, *246*, 6264−6268.
(43) Weiss, S. Measuring conformational dynamics of biomolecules by single molecule fluorescence spectroscopy. *Nat. Struct. Biol.* **2000**, *7*, 724−729.
(44) Ha, T.; Enderle, T.; Ogletree, D. F.; Chemla, D. S.; Selvin, P. R. et al. Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6264−6268.
(45) Schutz, G. J.; Trabesinger, W.; Schmidt, T. Direct observation of ligand colocalization on individual receptor molecules. *Biophys. J.* **1998**, *74*, 2223−2226.
(46) Ha, T.; Zhuang, X.; Kim, H.; Orr, J. W.; Williamson, J. R. et al. Ligand-induced conformational changes observed in single RNA molecules. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9077−9082.
(47) Pley, H. W.; Flaherty, K. M.; McKay, D. B. Three-dimensional structure of a hammerhead ribozyme. *Nature* **1994**, *372*, 68−74.
(48) Scott, W. G.; Finch, J. T.; Klug, A. The crystal structure of an all-RNA hammerhead ribozyme: a proposed mechanism for RNA catalytic cleavage. *Cell* **1995**, *81*, 991−1002.
(49) Ferre-D'Amare, A. R.; Zhou, K.; Doudna, J. A. Crystal Structure of a Hepatitis Delta Virus Ribozyme. *Nature* **1998**, *395*, 567−574.
(50) Rupert, P. B.; Ferre-D'Amare, A. R. Crystal structure of a hairpin ribozyme-inhibitor complex with implications for catalysis. *Nature* **2001**, *410*, 780−6.
(51) Rupert, P. B.; Massey, A. P.; Sigurdsson, S. T.; Ferre-D'Amare, A. R. Transition state stabilization by a catalytic RNA. *Science* **2002**, *298*, 1421−1424.
(52) Walter, N. G.; Burke, J. M. The hairpin ribozyme: structure, assembly and catalysis. *Curr. Opin. Chem. Biol.* **1998**, *2*, 24−30.
(53) Fedor, M. J. Structure and function of the hairpin ribozyme. *J. Mol. Biol.* **2000**, *297*, 269−91.
(54) Walter, N. G.; Burke, J. M. Real-time monitoring of hairpin ribozyme kinetics through base-specific quenching of fluorescein-labeled substrates. *RNA* **1997**, *3*, 392−404.
(55) Walter, N. G.; Burke, J. M.; Millar, D. P. Stability of hairpin ribozyme tertiary structure is governed by the interdomain junction. *Nat. Struct. Biol.* **1999**, *6*, 544−9.
(56) Okumus, B.; Ha, T. How intrinsic are molecular heterogeneities observed in single molecule measurements?: Vesicle encapsulation studies. *Biophys. J.* **2004**, *86*, 601A−610A.
(57) Lu, H. P.; Xun, L. Y.; Xie, X. S. Single-molecule enzymatic dynamics. *Science* **1998**, *282*, 1877−1882.
(58) Edman, L.; Rigler, R. Memory landscapes of single-enzyme molecules. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8266−8271.
(59) Fersht, A. *Structure and Mechanism in Protein Science*; Freeman: New York, 1999.
(60) Cai, Z. P.; Tinoco, I. Solution structure of loop a from the hairpin ribozyme from tobacco ringspot virus satellite. *Biochemistry* **1996**, *35*, 6026−6036.
(61) Butcher, S. E.; Allain, F. H. T.; Feigon, J. Solution structure of the loop B domain from the hairpin ribozyme. *Nat. Struct. Biol.* **1999**, *6*, 212−216.
(62) Pan, J.; Woodson, S. A. The effect of long-range loop-loop interactions on folding of the Tetrahymena self-splicng RNA. *J. Mol. Biol.* **1999**, *294*, 955−965.
(63) Herschlag, D. RNA chaperones and the RNA folding problem. *J. Biol. Chem.* **1995**, *270*, 20871−20874.
(64) Weeks, K. M. Protein-facilitated RNA folding. *Curr. Opin. Struct. Biol.* **1997**, *7*, 336−342.
(65) Hsien, J.; Andrews, A. J.; Ferike, C. A. Roles of protein subunit in RNA-protein complexes: Lessons from Ribonuclease P. *Biopolymers* **2004**, *73*, 79−89.
(66) Sorin, E. J.; Rhee, Y. M.; Nakatani, B. J.; Pande, V. S. Insights into nucleic acid conformational dynamics from massively parallel stochastic simulations. *Biophys. J.* **2003**, *85*, 790−803.
(67) Nivon, L. G.; Shakhnovich, E. I. All-atom Monte Carlo simulation of GCAA RNA folding. *J. Mol. Biol.* **2004**, *344*, 29−45.

AR040142O