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Structural rearrangements linked to global folding pathways of the *Azoarcus* group I ribozyme

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Summary

Stable RNAs must fold into specific three-dimensional structures to be biologically active, yet many RNAs form metastable structures that compete with the native state. Our previous time-resolved footprinting experiments showed that *Azoarcus* group I ribozyme forms its tertiary structure rapidly ($\tau < 30$ ms) without becoming significantly trapped in kinetic intermediates. Here we use stopped-flow fluorescence spectroscopy to probe the global folding kinetics of a ribozyme containing 2-aminopurine in the loop of P9. The modified ribozyme was catalytically active and exhibited two equilibrium folding transitions centered at 0.3 and 1.6 mM Mg^{2+} , consistent with previous results. Stopped-flow fluorescence revealed four kinetic folding transitions with observed rate constants of 100, 34, 1, and 0.1 s^{-1} at 37 °C. From comparison with time-resolved Fe(II)-EDTA footprinting of the modified ribozyme under the same conditions, these folding transitions were assigned to formation of the I_C intermediate, tertiary folding and docking of the nicked P9 tetraloop, reorganization of the P3 pseudoknot, and refolding of non-native conformers, respectively. The footprinting results show that 50–60% of the modified ribozyme folds in less than 30 ms, while the rest of the RNA population undergoes slow structural rearrangements that control the global folding rate. The results show how small perturbations to the structure of the RNA, such as a nick in P9, populate kinetic folding intermediates that are not observed in the natural ribozyme.

Keywords

RNA folding; ribozyme; stopped-flow fluorescence; time-resolved footprinting; 2-aminopurine

Introduction

The formation of tertiary structure allows RNAs to function efficiently as catalysts, aptamers and switches ^{1;2;3}. While some RNAs fold in 10–100 ms, other RNA sequences become kinetically trapped in alternative conformations that persist for 10–100 seconds or longer ^{4;5}; ⁶. Theoretical models and experimental results suggest that the partitioning of the RNA population between folding pathways that lead to the native structure or to an alternative metastable structure occurs early in the folding pathway ⁷. Therefore, it is important to

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understand which interactions determine the folding kinetics of the RNA and the uniqueness of the folding pathway.

The group I ribozyme from pre-tRNA^{ile} of the bacterium *Azoarcus* sp. BH72 forms a stable structure⁸ and folds rapidly at 37-50 °C without significantly becoming trapped in metastable kinetic intermediates^{9;10}. The equilibrium folding pathway in Mg²⁺ involves at least two macroscopic transitions (Scheme I). In the U → I_C transition, which occurs around 0.2-0.3 mM MgCl₂, the core helices are assembled and the RNA collapses into native-like intermediates (I_C) with similar dimensions as the native RNA¹¹. Small angle X-ray scattering studies showed that tertiary interactions between helices stabilize I_C, although solvent can still penetrate the interior of the ribozyme¹². In the I_C → N transition, which occurs in 2-3 mM MgCl₂, the native tertiary interactions between helices are fully established and the RNA becomes catalytically active.

Using time-resolved hydroxyl radical footprinting, which reveals the backbone accessibility of individual riboses, we found that the wild type *Azoarcus* ribozyme folds to its native tertiary structure (N) in 5-20 ms at 37 °C¹⁰. To study the global folding kinetics of the *Azoarcus* ribozyme, we tagged a tetraloop in the ribozyme with the fluorescent adenine analog 2-aminopurine (2-AP)¹³. 2-AP forms an isosteric Watson-Crick type base pair with uracil, and thus causes minimal perturbation to RNA secondary structure. It has been widely used to study conformational changes in nucleic acids¹⁴ because the quantum yield of the fluorescence is sensitive to the local $\pi \rightarrow \pi^*$ stacking interactions with neighboring nucleobases^{13;15}.

Here, we find that the change in the fluorescence of a 2-AP-tagged ribozyme is consistent with the Mg²⁺-dependent equilibrium folding pathway previously deduced from other biochemical and biophysical methods^{9;11;12}. Analysis of the folding kinetics with a stopped-flow spectrofluorometer showed that the intermediate forms with $\tau \leq 10$ ms and that the tertiary folding occurs with $\tau \leq 30$ ms, consistent with the results of time-resolved hydroxyl radical footprinting experiments. However, the folding kinetics of the tagged ribozyme reveals at least two additional steps in the folding process. We attribute one of these additional steps to refolding of the P3 pseudoknot in a sub-population of non-native intermediates. A second slow transition is assigned to reorganization of the ribozyme core after the P3 pseudoknot is formed.

Results

Fluorophore-tagged *Azoarcus* ribozyme

The native state of the *Azoarcus* ribozyme is stabilized by docking of the GAAA tetraloop in paired region P9 into a receptor in P5/P5a^{8;16;17}. We previously found that this interaction also stabilizes the compact folding intermediates (I_C) of the ribozyme¹². Thus, tetraloop-receptor docking is expected to report the folded state of the ribozyme. To monitor the folding transitions in the ribozyme, the third base in the GAAA tetraloop (A191) was replaced by 2-AP (Figure 1a). Tetraloop docking involves a network of hydrogen bonding and additional base stacking interactions between purines in the P9 loop and bases in the receptor (Figure 1b)¹⁸. Therefore, the fluorescence emission of 2-AP191 is expected to be perturbed in the native ribozyme, relative to the undocked tetraloop.

The desired 2-AP substitution at A191 was introduced by annealing a synthetic 14-nt RNA (OP9G) containing the 3' end of the ribozyme (2-AP or A191 to G205) with a 187-nt T7 transcript (IΔP9) that provides the rest of the ribozyme (G4 to A190) (Figure 1a). This strategy was successfully used for crystallization of the *Azoarcus* splicing complex¹⁷ and modification of a group II ribozyme¹⁹. Cleavage of the 5' splice site and hydroxyl radical footprinting of the folded RNA demonstrated that the 2-AP-labeled ribozyme complex was active and formed

the expected tertiary contacts (Figure S1 in Supporting Material). The results also confirmed that the $\Delta P9$ RNA does not fold correctly without the OP9G oligonucleotide.

To determine the best conditions for folding experiments, the binding affinity of the fluorescent oligonucleotide to the $\Delta P9$ transcript was measured by a gel-mobility shift assay (Figure S2a). In the unfolded state (10 mM Tris-HCl buffer), the dissociation constant (K_d) of OP9G was $1.7 \pm 0.8 \mu\text{M}$. The addition of 0.5 mM Mg^{2+} or 50 mM Na^+ to the annealing buffer significantly decreased the K_d to 89 ± 6 and 490 ± 30 nM, respectively (Figure S2b). Consequently, RNA ($\geq 2 \mu\text{M}$) was initially annealed in 0.5 mM Mg^{2+} for kinetic folding experiments to ensure that all of the fluorescent oligomer was bound. However, as folding intermediates (I_C) of the *Azoarcus* ribozyme form in sub-millimolar magnesium^{9;11}, our kinetic folding experiments start from a mixture of I_C and U, which may contribute to the heterogeneity of the observed time constants as described below.

Equilibrium folding monitored by steady-state fluorescence

To determine if the change in fluorescence intensity of 2-AP reports the known folding transitions of the *Azoarcus* ribozyme, the steady-state fluorescence of the $\Delta P9$ •OP9 complex was measured over a range of Mg^{2+} concentrations (Figure 2a). Two transitions were observed when Mg^{2+} was added to the $\Delta P9$ •OP9 complex. In the first phase, the 2-AP fluorescence signal increased with Mg^{2+} up to 0.5 mM. This was followed by a cooperative decrease in fluorescence intensity upon further addition of Mg^{2+} up to 20 mM (Figure 2b). As expected, the fluorescence intensity of the OP9G oligomer alone changed negligibly under these conditions.

The change in relative 2-AP fluorescence was fit to a three-state cooperative binding model for Mg^{2+} , as described in Materials and Methods (Figure 2b). The populations of U, I_C and N states predicted from the fits (Figure 3a) corresponded to a cooperative loss of unfolded RNA with an apparent midpoint of $C_{m,I} = 0.27 \pm 0.04$ mM and Hill coefficient of $n_{H,I} = 5 \pm 2$, and an increase in native RNA with $C_{m,N} = 1.6 \pm 0.2$ mM and $n_{H,N} = 1.6 \pm 0.3$ (Figure 3a).

These parameters agreed closely with those obtained by other biophysical and biochemical methods (Figure 3b and Table 1), confirming that the 2-AP fluorescence reports the same folding transitions detected previously¹². The midpoint of the first folding transition agreed, within experimental error, with midpoints for the decrease in the radius of gyration (R_g) measured by SAXS (0.34 mM) and the protection from ribonuclease T1 digestion (0.2 – 1.1 mM) (Table 1), which both report the transition from U $\rightarrow$ I_C . The midpoint of the second fluorescence transition overlapped the $I_C \rightarrow$ N tertiary folding transition, as monitored by hydroxyl-radical cleavage ($C_m = 0.7 - 1.8$ mM) and the onset of catalytic activity ($C_m = 2.0$ mM) (Figure 3b). Thus, the change in fluorescence reflects Mg^{2+} -induced folding of the *Azoarcus* ribozyme, and the folding equilibrium is not severely perturbed by the modification.

The increase in 2AP fluorescence during the U $\rightarrow$ I_C folding transition may be due to improved hybridization of the OP9G oligonucleotide, or a change in the environment of the 2-AP in the P9 tetraloop in the collapsed state. The decrease in 2-AP fluorescence in the $I_C \rightarrow$ N step at higher Mg^{2+} is probably due to stacking of 2-AP with bases in the P5/5a receptor. There is precedence for different folding steps having opposite effects on the environment of the fluorophore probe. For example, the fluorescein labeled catalytic domain of RNase P exhibits a decrease and increase in fluorescence signal during its U $\rightarrow$ I_{eq} and $I_{eq} \rightarrow$ N folding transitions, respectively²⁰.

Ribozyme core mutation perturbs folding monitored by fluorescence

To verify that the fluorescence of 2-AP191 reports global folding and not just local perturbation of the tetraloop, we repeated the equilibrium folding experiments with a ribozyme containing the mutation G44C in the P3 pseudoknot within the ribozyme core. Disruption of P3 by this base substitution destabilizes both the compact intermediate (I_C) and the native state (N) of the *Azoarcus* ribozyme¹².

The same changes in fluorescence intensity were observed, but much more Mg^{2+} was needed to fold the P3 mutant RNA (Figure 2b). Although the midpoint of the first $U \rightarrow I_C$ transition ($C_{m,I} = 0.32 \pm 0.01$ mM) was only slightly higher for the P3 mutant than for the wild type ribozyme, the midpoint of the second $I_C \rightarrow N$ transition was ten times higher ($C_{m,N} = 17 \pm 2$ mM) than for the wild-type (Figure 2b). Moreover, the P3 2-AP-tagged RNA did not fold completely, even in 50 mM $MgCl_2$. This observation is consistent with the larger R_g and reduced catalytic activity (40%) of the P3 ribozyme observed previously¹². Thus, the change in fluorescence reflects the global structure of the RNA and not just changes in the local environment of the probe.

Folding kinetics of the *Azoarcus* ribozyme by time-resolved fluorescence

The *Azoarcus* ribozyme forms its native 3D structure in $\tau \sim 10$ -30 ms, with 10-20% of the wild type population becoming kinetically trapped in misfolded intermediates at 37 °C¹⁰. As the $U \rightarrow I_C$ and $I_C \rightarrow N$ transitions produce opposite changes in 2AP fluorescence, we used stopped-flow fluorescence spectroscopy to capture the kinetics of both steps. The folding kinetics of the *Azoarcus* ribozyme was monitored by mixing the annealed $\Delta P9 \bullet OP9G$ complex with $MgCl_2$ -containing buffer in a stopped-flow spectrofluorometer (see Materials & Methods). To ensure that the folding rate was not limited by binding of OP9G, a large excess of (L-3) $\Delta P9$ RNA was used (typically 10 μM). Above 4 μM RNA, the observed folding rates are independent of RNA concentration (Figure S3).

Under native conditions (15 mM Mg^{2+}), we observed at least four kinetic phases within the first 10 s of each trajectory. A fast increase in fluorescence ($k_{obs,1} = 106$ s⁻¹) was followed by two slower phases of decreasing fluorescence ($k_{obs,2} = 34$ s⁻¹; $k_{obs,3} = 1$ s⁻¹) (Figure 4a, inset). When the ribozyme was allowed to fold for a longer period of time (10 s), an additional slow decrease in fluorescence appeared, with an apparent rate constant, $k_{obs,4} = 0.1$ -0.3 s⁻¹ (Figure 4a). A comparison of three- and four-phase fits to the entire trajectory confirmed the presence of four qualitatively distinct kinetic transitions between 10⁻³ and 10 s (Figure S4). As we describe below, this complexity arises from the two equilibrium folding transitions ($U \rightarrow I_C \rightarrow N$), and from partitioning of the tagged ribozyme complex among folding pathways that include misfolded intermediates.

The rapid increase in fluorescence (≥ 100 s⁻¹) correlates with the rate of compaction of the *Azoarcus* ribozyme measured by stopped-flow SAXS (J. H. Roh, personal communication) and was assigned to the $U \rightarrow I_C$ step. Because I_C is already partially populated at the start of the experiment and this step occurs very near the deadtime of our instrument, 100 s⁻¹ represents the lower limit of the $U \rightarrow I_C$ rate constant. The apparent rate constant of the second phase (34 s⁻¹) is close to the lower limits of the folding rate observed by time-resolved hydroxyl radical cleavage (30-100 s⁻¹)¹⁰ (Table 2). This, and the correlation between a decrease in fluorescence and population of the native state in the equilibrium Mg^{2+} titrations (Figure 3), led us to assign the second kinetic phase to a tertiary folding transition ($I_C \rightarrow N$) of the *Azoarcus* ribozyme.

The third phase (~ 1 s⁻¹), in which the fluorescence continues to decrease, either represents an additional structural rearrangement that was not detected in previous footprinting experiments, or molecules in the RNA population that fold via a separate (parallel) transition state ensemble.

Finally, the slowest decrease in fluorescence ($k_{obs,4} = 0.12 \text{ s}^{-1}$) most likely corresponds to a reorganization of the ribozyme tertiary structure linked to the conformation of the active site, as it was only detected when the labeled OP9G oligonucleotide included the ribozyme's penultimate G205 (Figure 4a). When the 0.5 s and 10 s data were appropriately scaled and concatenated, the slowest kinetic phase accounted for ~45% of the total decrease in fluorescence.

Thus, the sensitivity of the fluorescence probe reveals additional kinetic folding transitions in the *Azoarcus* ribozyme that are not visible in the equilibrium folding pathway, and the enormous range of the folding dynamics ($\leq 10 \text{ ms}$ to $\geq 10 \text{ s}$). Below, we use time-resolved footprinting to show that the slow steps arise from misfolded intermediates that become populated when P9 is nicked and modified, explaining why these slow transitions were not detected in previous experiments with the single-transcript ribozyme¹⁰.

P3 mutant delays rapid folding kinetics of the *Azoarcus* ribozyme

To determine if the folding kinetics of the *Azoarcus* ribozyme is sensitive to the stability of the tertiary structure, stopped-flow fluorescence experiments were also carried out with the 2AP-tagged P3 mutant (G44C). Unlike the wild-type RNA, the P3 mutant ribozyme showed three kinetic phases: a fast increase in fluorescence intensity (115 s^{-1}) followed by a slower increase in fluorescence (5 s^{-1}) (Figure 4b) and finally an extremely slow decrease in fluorescence ($\sim 0.01 \text{ s}^{-1}$) that was only observed at high Mg^{2+} . Our previous SAXS studies¹² and the steady-state fluorescence measurements showed that the P3 mutant undergoes a collapse transition but produces an intermediate that is less compact than the wild type intermediates. The non-native conformation of the intermediates and the marginal stability of the native structure could explain why the P3 mutant takes much longer to fold (0.01 s^{-1}) than the wild type RNA (30 s^{-1}). Thus, destabilization of the core P3 pseudoknot not only alters the average conformation of the I and N states but also significantly delays formation of the tertiary structure, reflecting a tight correlation between the thermodynamic stability of the RNA tertiary structure and the speed and accuracy of the RNA folding process.

Temperature-dependent folding kinetics of the *Azoarcus* ribozyme

The activation energy for the tertiary folding of the wild type 2AP-tagged ribozyme was determined from the temperature-dependence of its folding kinetics. The annealed (L-3) IAP9RNA•OP9G complex was pre-equilibrated at each temperature for 10 min, before the folding reaction was started by addition of 15 mM MgCl_2 (Figure 5a). Individual traces were collected for 0.5 s and 10 s in order to cover the full time window of the folding reactions, and the average rate constants plotted as a function of temperature, from 20 to 50 °C (Figure 5b). Because the transitions were well separated in time, it was possible to assign each kinetic phase of the fluorescence signal to a separate relaxation step over most of the temperature range.

The activation energies for each phase were $E_{a,1} = 16 \text{ kcal/mol}$; $E_{a,2} = 35 \text{ kcal/mol}$, $E_{a,3} = 19 \text{ kcal/mol}$ and $E_{a,4} = 9 \text{ kcal/mol}$ (Figure 5b). These values are within the range reported for other RNA folding reactions, such as folding of the full-length *Tetrahymena* intron (21 kcal/mol)^{21;22;23}, formation of mRNA pseudoknot (34 kcal/mol)²⁴ and folding of catalytic domain of RNase P (32-36 kcal/mol)²⁰. Thus, most steps are associated with a significant transition state enthalpy, consistent with changes in base stacking interactions and hydrogen bonds. The smallest activation energy (9 kcal/mol) for the slowest kinetic phase points to an entropic barrier for the conformational rearrangement associated with the penultimate G205. An entropic barrier was previously reported for the docking of a pyrene-labeled substrate in the *Tetrahymena* ribozyme, and may be a signature for structural rearrangements within the active site of group I ribozymes²⁵.

Magnesium-dependent folding kinetics of the *Azoarcus* ribozyme

To further elucidate the origin of the complex folding dynamics of the *Azoarcus* ribozyme, the magnesium-dependence of the folding kinetics was determined as described above (Figure 6). If the ribozyme follows a simple two-state folding reaction, then the folding and unfolding rate constants should depend exponentially on Mg^{2+} concentration, producing a “chevron” profile as observed in the dependence of protein folding kinetics on denaturant²⁶. Since the folding mechanism of the *Azoarcus* ribozyme is more complex, it is expected to deviate from this simple scenario²⁷. Nonetheless, we found that observed rate constant for each phase increased with Mg^{2+} concentration above the midpoint of the transition (C_m), as expected if the folding rate depends on the stability of the folded RNA (Figure 6).

If, like the folding free energy, the transition state free energy $\Delta G^\ddagger$ is assumed to vary linearly with $\ln C$ ²⁸, then the forward or reverse rate constant for each step can be expressed as

$\ln k = \ln k_o - \Delta G^\ddagger / RT = \ln k_o - \Delta G_{\text{ref}}^\ddagger / RT + m^\ddagger \ln C$, in which k_o is the folding rate when $\Delta G^\ddagger = 0$, and $\Delta G_{\text{ref}}^\ddagger$ is the transition state free energy in 1 mM MgCl_2 . This simple model fit the kinetic data well, within the error of the observed rate constants (Figure 6). The dependence of $\Delta G^\ddagger$ on Mg^{2+} ($\ln C$) ranged from 0.3 to 0.6 kcal/mol at 37 °C for the various rate constants, about half the magnitude of -1.0 kcal/mol for the equilibrium between (I_C and N) (Figure 3b). This suggests that the transition state ensembles are less structured than the native state.

Fast Fenton footprinting reveals reorganization of the ribozyme core

The stopped-flow fluorescence data show that about 45% of the fluorescent signal associated with folding occurs slowly, over 10 s. On the other hand, previously time-resolved hydroxyl radical footprinting showed that tertiary interactions throughout the wild type ribozyme formed in 5-20 ms¹⁰. Therefore, the slow changes in 2-AP fluorescence might represent conformational steps that are not detected by hydroxyl radical footprinting. Alternatively, the slow decrease in 2-AP fluorescence might reflect new intermediates caused by the nick in the P9 tetraloop, which is critical for the stability of the ribozyme core²⁹. To distinguish between these possibilities and assign the fluorescent signals to specific structural changes in the RNA, we carried out time-resolved footprinting on the $I\Delta P9$ -OP9 complex under conditions as similar as possible to those used for the stopped-flow fluorescence experiments (Figure S5).

The $I\Delta P9$ -OP9G complex was pre-formed in 0.5 mM MgCl_2 and then rapidly mixed with MgCl_2 (15 mM final) at 37 °C to begin the folding reaction. The tertiary structure of the RNA was probed using the fast Fenton reaction method, which resolves conformational changes within 5 ms³⁰. Unlike the unimolecular ribozyme, the backbone of the $I\Delta P9$ -OP9G complex was protected in two or three stages, indicating the presence of tertiary folding intermediates (Figure 7; Table S1). Since the protection patterns of the fully-folded $I\Delta P9$ -OP9G and unimolecular ribozymes match closely, the change in the observed folding rates had to come from a change in the folding pathway of the $I\Delta P9$ -OP9G ribozyme.

First, we observed that all of the expected segments of the $I\Delta P9$ -OP9G backbone were rapidly protected from hydroxyl radical cleavage, with transition midpoints shorter than 5 ms ($k_{\text{obs}} \geq 100 \text{ s}^{-1}$) (Figure 7a,b). On average, the backbone protections were 40-60% saturated relative to the native state within this initial period, consistent with the fraction of rapidly folding RNA obtained from fluorescence studies. Thus, the tertiary interactions form rapidly in the 2-AP-labeled ribozyme, but to a lesser extent than in the unimolecular ribozyme.

The rate of backbone protection was slightly faster than the apparent folding rate monitored by fluorescence ($k_2 = 34 \text{ s}^{-1}$). Fluorescence emission reports the microenvironment of 2-AP at position 191, and its slower rate of change may reflect weaker docking of the nicked P9 tetraloop with its receptor. The P5/5a receptor for the P9 tetraloop (nt 62) was protected more

slowly (20 s^{-1}) than adjacent residues, and to a smaller degree (25-30%). Thus, the footprinting results support the interpretation that the docked P9 complex is less stable or more dynamic than expected.

Second, we used the footprinting data to obtain a more detailed explanation for the slow steps of folding, which account for about half of the dynamic change in the RNA (Figure 7c). In the ribozyme core, the slow rate constants for protection of P4, P6, J6/6a, and P3 overlapped with each other, in the range of $0.15\text{--}0.5 \text{ s}^{-1}$. Residues involved in other tertiary interactions saturated even more slowly ($0.02\text{--}0.08 \text{ s}^{-1}$). The similarity between these values and the slowest phase of fluorescence emission decay (k_4), which accounts for 40-45% of the population, strongly supports the assumption that this step arises from reorganization of the RNA tertiary (and secondary) structure. We cannot judge, however, whether this reorganization is a concerted motion throughout the ribozyme core, or a series of local movements with slightly different rate constants, for which fluorescence can only report an average rate.

Unlike residues elsewhere in the structure, the slow rate constants for the protection of J6/6a (nt 95-99) and P3 (nt 137-141) could not be precisely determined. Instead, probable solutions to the rate equations covered the range of $0.5\text{--}5 \text{ s}^{-1}$. As these two regions interact in an A-minor motif in the native ribozyme¹⁷, it is unlikely that this indetermination is simply a result of random noise in the data. Rather, this spread in the probable values of the rate constants may be due to averaging of two underlying processes with different rates. We speculate that P3 and J6/6a, or the pseudoknot belt that partially covers P3 and P6, undergo a structural change at a rate of $1\text{--}5 \text{ s}^{-1}$ that is responsible for the fluorescence decrease at 1 s^{-1} (k_3). These residues are additionally protected by the slow reorganization of folding intermediates at 0.1 s^{-1} (k_4), either subsequent to the preceding step or in parallel to it.

This model is further supported by direct evidence for refolding of the P3 pseudoknot from the time-resolved footprinting data (Figure 7c). The pseudoknot “belt” (nt 39-54) is normally exposed to solvent in the native ribozyme⁹, except for nt 49-50 that form part of the central triple helix (J3/4). In our experiments, however, nt 43-52 were rapidly protected from cleavage ($k \geq 100 \text{ s}^{-1}$), then exposed with an average rate constant of $5.4 \pm 0.8 \text{ s}^{-1}$ (Table S2). Therefore, the decay rate of 1 s^{-1} in the fluorescence data most likely captures refolding of misfolded intermediates lacking the P3 pseudoknot. Residues 49-50 showed the same behavior, but the extent of protection slowly increased again after 1 second with a rate constant of $k \sim 0.3 \text{ s}^{-1}$ (Figure 7b). In the fully-folded RNA, nt 49-50 are protected from hydroxyl radical cleavage as expected.

Discussion

The concordance of multiple structural probes can reveal new features of RNA folding pathways (e.g., 31³²). In our study, a side-by-side comparison of stopped-flow fluorescence and time-resolved footprinting measurements allowed the collective behavior of individual residues to be correlated with global steps in the folding pathway. Stringent statistical analysis of the rate constants revealed steps in the refolding pathway that were not initially obvious. In addition, the shorter dead time of the stopped-flow spectrometer (1.8 ms vs. 5 ms for footprinting) demonstrated that the earliest folding transitions of the *Azoarcus* ribozyme occur in $\leq 10 \text{ ms}$ at 37°C .

As expected from previous work⁹, the 2-AP labeled ribozyme exhibited two distinct equilibrium folding transitions as a function of Mg^{2+} concentration. The first transition appeared at the same Mg^{2+} concentration as the $\text{U} \rightarrow \text{I}_\text{C}$ collapse transition monitored by SAXS and other methods¹¹. By contrast, the Mg^{2+} requirement for the second transition is in good agreement with the midpoint of the $\text{I}_\text{C} \rightarrow \text{N}$ tertiary folding transition monitored using

biochemical probes. That the fluorescence transitions in the P3 mutant ribozyme occurred at higher magnesium concentrations further supports the conclusion that the change in fluorescence emission represents global folding transitions rather than local perturbations to the probe environment. Additional folding transitions may overlap these two main phase transitions, such as a non-sequence specific contraction of the RNA in low Mg^{2+} . Similarly, catalytic activity increases more cooperatively with respect to Mg^{2+} than backbone accessibility or fluorescence. Thus, formation of the native RNA may involve two overlapping transitions leading to a folded, inactive RNA (I_F) and to the active ribozyme (N) ³³.

The folding kinetics of the 2-AP-labeled *Azoarcus* ribozyme were unexpectedly complex, with at least four time-dependent transitions (Figure 8). The multi-stage folding kinetics observed by stopped-flow fluorescence can either be explained by partitioning of the population among parallel folding pathways with different transition states, or by sequential changes in the structure of the RNA which each produce a change in the environment of the fluorophore. However, the immediate partial saturation of backbone contacts everywhere in the RNA is most easily explained if part of the RNA population folds very rapidly. Thus, the results of the footprinting experiments are most consistent with partitioning among fast and slow folding pathways (Figure 8).

In our model, we propose that collapse and the initial formation of tertiary interactions both occur within 10 ms in 15 mM $MgCl_2$. These steps are not well resolved in our experiments, especially as the folding trajectories begin with a mixture of U and I_C in 0.5 mM $MgCl_2$. For about half the $I\Delta P9 \bullet OP9G$ ribozyme population, these transitions lead directly to the native tertiary structure. Partial protection of the 5' side of P3 (nt 43-52), which is exposed to solvent in the native ribozyme, suggests that non-native intermediates are also formed within this time. Both the fluorescence and footprinting results suggest that in the $I\Delta P9 \bullet OP9G$ complex, docking of P9 lags behind tertiary interactions in the rest of the RNA ($34 s^{-1}$). This fluorescence signal may alternatively represent a local conformational change in the P9 tetraloop, but the activation energy of this step (35 kcal/mol•K) is more consistent with a larger structural rearrangement.

Next, we propose that the slow folding steps arise from RNA molecules that initially fail to form the P3 pseudoknot during the collapse transition. The P3 pseudoknot is universally conserved among group I ribozymes and required for ribozyme activity ³⁴. Mispairing of P3 causes the *Tetrahymena* ribozyme to become kinetically trapped in metastable folding intermediates in vitro ³⁵, and such a possibility for the *Azoarcus* ribozyme was suggested by previous ribonuclease probing studies on the pre-tRNA and predicted secondary structures ^{36;37}.

The misfolded intermediates of the *Azoarcus* ribozyme are resolved in at least two steps: refolding of the interface between P3, P4 and P6 at $1-5 s^{-1}$ which exposes residues in P3, and the formation of core tertiary interactions at $\sim 0.1 s^{-1}$ or slower. The burial, exposure and rebury of residues in J3/4 may be explained by either sequential or parallel folding routes, although for the reasons given above we favor a parallel model (Figure 8). The slowest kinetic step may include a rearrangement of the G-binding pocket in the active site, similar to that reported for the *Tetrahymena* ribozyme ^{38;39}.

In conclusion, the *in vitro* refolding rates of ribozymes vary dramatically, with some sequences achieving their native structure within 10 ms and others requiring 100 s or longer ^{4;5}. This variation in folding times can be explained by the kinetic partitioning of RNA populations among native-like and non-native folding intermediates, as the latter require long times to refold. The combination of fluorescence and hydroxyl radical footprinting corroborate our previous findings that the tertiary structure of the *Azoarcus* ribozyme forms rapidly (~ 10 ms) in 10-15 mM $MgCl_2$ once the secondary structure of the RNA has been established ^{9;10}. The

Azoarcus ribozyme is unusually stable⁸, and this may explain why a large fraction of the population appears to fold directly to the native state.

We previously found that single base substitutions in the P9 tetraloop that destabilize the ribozyme's tertiary structure also lowered the fraction of fast-folding RNA, by diverting a larger portion of the population through metastable folding intermediates¹⁰. Here, we observe that a nick in P9 has a similar effect on the proportions of native-like and non-native folding intermediates. In both cases, perturbations to P9 affect folding of the entire RNA, consistent with the importance of these interactions in group I introns⁴⁰. That even small perturbations to the tertiary structure increase the heterogeneity of the folding dynamics illustrates the tight connection between the thermodynamic stability of the tertiary interactions and the accuracy of the folding process¹⁰. This correlation has important consequences for the evolution of RNA sequences, and for the assembly of large ribonucleoprotein complexes.

Materials and Methods

Plasmids and RNA preparation

Plasmids encoding (L-3) Δ P9 ribozyme with wild-type core or containing the P3 mutation G44C were prepared by PCR amplification of pAz-IVS and pAzG44CIVS, respectively, using the downstream primer 5'CGC CTG CAG AAA CTC TTC ATC GCC ACT CCC TGG ACT AT 3', and upstream primer 5'CGC GAA TTC TAA TAC GAC TCA CTA TAG GCG ATG TGC CTT GCG CCG GGA 3'. The primers contain *Eco*R I and *Pst* I restriction sites insertion into pUC18.

The (L-3) Δ P9 ribozyme was transcribed *in vitro* with T7 RNA polymerase from plasmid DNA digested to completion with *Ear* I. Transcription reactions (10 mL) were carried out in disposable 50 mL centrifuge tubes and purified by denaturing 4% (w/v) PAGE as described earlier¹². The eluate was pooled and exchanged 4 times with 10 mM Tris-HCl (pH 7.5) using Centricon Plus-30 concentrators (Amicon) at 4 °C. The concentration of (L-3) Δ P9 RNA was determined using absorption at 260 nm (extinction coefficient of $1.82 \cdot 10^6$ L/mol•cm).

Preparation of fluorescently labeled oligonucleotides

The OP9G fluorescent oligonucleotide (5' r(2AP) AGC CAC ACA AAC CG 3') was obtained from Dharmacon (Boulder, CO). OP9G anneals to the 3' end of the Δ P9 ribozyme fragment so that 2-AP replaces A191. U194 was replaced with C to increase the stability of the complex¹⁷. The oligonucleotide was deprotected and purified on a denaturing 15% polyacrylamide gel following the manufacturer's protocols. The purified RNA was resuspended in 10 mM Tris-HCl (pH 7.5) and the concentration estimated using $\epsilon_{260} = 1.39 \cdot 10^5$ L/mol•cm.

Oligonucleotide gel-shift experiments

OP9G oligonucleotide was 5'-labeled with ³²P and gel purified using standard methods. 15 nM radiolabeled oligomer was annealed with 0 – 20 μ M (L-3) Δ P9 RNA in 10 mM Tris-HCl (pH 7.5), in 10 μ L reaction volume, for 2 min at 65 °C followed by 1 min on ice. Samples (2 μ L) were loaded on a 10% (w/v) native polyacrylamide gel (29:1 acrylamide/bis, 34 mM Tris, 66 mM Hepes, 0.1 mM EDTA, 0.5 mM MgCl₂) at 4°C and electrophoresed at 15 W for 1.5 hours. Dried gels were quantified using a Molecular Dynamics PhosphorImager. The fraction of oligomer bound to (L-3) Δ P9 RNA, f_B , was determined from the counts in the shifted bands (B) relative to the total counts (B + F) in the lane, and normalized to the extent of binding at saturation. The binding data were fit to a single-site binding isotherm to obtain the equilibrium dissociation constant.

Self-cleavage activity

DNA templates for T7 run-off transcription of RNAs starting 33 nt upstream of the 5' splice site and ending in G205 (5'ex-I) or A190 (5' ex-IΔP9) were constructed by PCR. Self-splicing reactions were carried out in 25 mM Na-HEPES as previously described⁸. For reactions with IΔP9, 20 nM uniformly labeled RNA was annealed to 0.5 μM OP9. Reactions were initiated with GTP (100 μM) and stopped with 30 mM EDTA.

Equilibrium fluorescence measurements

Steady-state fluorescence experiments were carried out on an Aviv ATF105 spectrofluorometer in a 500 μl quartz cuvette with 1 cm path length (Hellma Biotech). All equilibrium experiments were carried out in 10 mM Tris-HCl (pH 7.5) buffer. The fluorescently tagged *Azoarcus* RNA for the equilibrium measurements was prepared by annealing 2 μM OP9G oligonucleotide with 10 μM (L-3) IΔP9 RNA in 10 mM Tris-HCl (pH 7.5) at 65°C for 1 min, followed by slow cooling to room temperature. A five-fold excess of (L-3) IΔP9 RNA was used to ensure that all the 2-AP oligonucleotide was bound.

The annealed RNA was transferred to the cuvette for fluorescence measurements where the MgCl₂ was incrementally increased from 0 to 15 mM. The sample was incubated at 37°C for 5 min after each addition before taking the reading. The 2-AP excitation wavelength was set to 310 nm and the emission spectra were collected from 325-425 nm using 5 mm excitation and emission slit widths. The fluorescence intensity was recorded at the emission maximum of 2-AP (370 nm) and corrected for the small dilution caused by the titrant.

The emission intensity as a function of Mg²⁺ concentration, $F(Mg)$, was normalized using $F = [F(Mg) - F_{min}] / [F_{max} - F_{min}]$, in which F_{min} and F_{max} represent the minimum and maximum fluorescence signals over the Mg²⁺ titration. The relative fluorescence F was fit to a three-state model, $F = \chi_U F_U + \chi_I F_I + \chi_N F_N$, in which F_U , F_I and F_N are the fluorescence intensities of the U, I_C and N, respectively, and χ_i 's are the mole fraction of each species. For the wild type RNA, $F_U = 0.6$, $F_I = 1$ and $F_N = 0$. For the P3 mutant RNA, $F_U = 0$, $F_I = 1$ and $F_N = 0.3$. The mole fraction of each species was obtained from fits to eq. 1.

$$F = \frac{[F_U + F_I(C/C_I)^m + F_N(C/C_N)^n]}{[1 + (C/C_I)^m + (C/C_N)^n]} \quad (1)$$

The statistical weights of each term represent the free energy difference between the U and I_C or U and N states and are assumed to take the form $\Delta G = -RT(n \ln C - n \ln C_N)$, in which C is the Mg²⁺ concentration, C_I or C_N is the midpoint of each transition, and m and n represent the cooperativity of each transition with respect to Mg²⁺ concentration^{20,28}. Because the two transitions are well separated, the data were fit equally well by sequential two-state transitions (U to I_C and I_C to N), although this model slightly overestimates the concentration of I_C.

Stopped flow kinetic measurements

Time-dependent refolding experiments were carried out on an Applied Photophysics stopped-flow spectrofluorometer (SX.18MV) with a mixing dead time of 1.8 ms. The 2-AP-tagged ribozyme was prepared by annealing 4 μM U194C-OP9G with 20 μM (L-3) IΔP9 RNA in 10 mM Tris-HCl plus 0.5 mM Mg²⁺ at 65 °C for 5 min, followed by slow cooling to room temperature. The addition of 0.5 mM Mg²⁺ significantly increased the affinity of OP9G oligonucleotide for (L-3) IΔP9 RNA. In addition, a five-fold excess of (L-3) IΔP9 RNA was used to ensure that all the 2-AP oligonucleotide was bound. Folding reactions were initiated

by mixing equal volumes (100 μ l) of RNA solution and buffered MgCl_2 (0 - 15 mM final). Solutions were pre-equilibrated 10 min at 37 °C before mixing.

Excitation was at 310 nm and the change in fluorescence emission was measured using a wide band cut-off filter of 360 nm. At least six time traces with 1000 points per window were collected for each data set. The raw fluorescence intensities were normalized to the minimum and maximum signal observed during the course of each folding trajectory. The relative change in fluorescence recorded over 10 s and 500 ms were fit to $F = A_0 + A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t})$ and $F = A_0 + A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t}) + A_3(1 - e^{-k_3t})$, respectively. The observed rate constants for six traces were averaged. Data collected over 500 ms were used to measure the first two kinetic phases; data collected over 10 s provide better resolution of the last two kinetic phases. We could not accurately determine the amplitudes of the phases observed in different collection time windows. For certain conditions, data sets were concatenated by scaling the 500 ms data to the relative fluorescence at $t = 500$ ms in the 10 s data set. The amplitudes were estimated from multiphasic fits to the combined data to 10 s.

Time-resolved footprinting

(L-3) Δ P9 RNA (40 pmol) was 5' end-labeled with [^{32}P]-ATP and gel purified according to standard methods. Δ P9 RNA (50 nM) was annealed with 2 μM OP9G in 10 mM Na-cacodylate, 1 mM EDTA (CE) plus 0.5 mM MgCl_2 for 1 min at 65 °C, then placed on ice. For control reactions on completely folded RNA, MgCl_2 was added to 15 mM (final), and incubated 20 min at 50 °C. Time-resolved hydroxyl radical footprinting reactions were carried out with the Fenton reaction as previously described^{10;41}, except that the control software and protocols were slightly modified to optimize the RNA recovery.

The cleavage products in sequencing gels were quantified using SAFA (nts 24 to 104)⁴², or IMAGEQUANT (Molecular Dynamics). Five invariable products (residues 25, 64, 73, 74, 102) were used to standardize the band intensities⁴³. The extent and rates of protections were determined by normalizing relative saturation of each protection Y to the unfolded and folded controls (0 to 1, respectively) and fitting to first order rate equations as above. In parts of the sequence where high noise levels prevented using folded and unfolded controls for normalization, protections were scaled between maximum and minimum intensities.

Statistical analysis of parameters

To assess the reliability of kinetics parameters obtained from footprinting experiments, each transition was subjected to a bootstrap analysis⁴⁴ with 5000 resampling of fit residuals performed within MATLAB (Mathworks, Natick, MA). Outlier values were detected and removed by the method of Tukey⁴⁵ with boundaries set at 1.5 interquartile region distances from first and third quartiles. The resultant distributions were used to obtain a symmetric region around most probable value (mode) encompassing 95% of observations. When the noise in the data resulted in more than one significant peak in the parameter distribution (e.g. residues 137-141), the corresponding fits were treated independently to obtain different but equally probable sets of amplitudes and rates constants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. (L-3) *Azoarcus* ribozyme labeled with 2-aminopurine

(a) The fluorescent ribozyme was reconstituted by annealing (L-3) IΔP9 (grey) with OP9G oligomer (black) in 10 mM Tris buffer (pH = 7.5) at 65 °C for 2 min. A191 (red) was replaced by 2-aminopurine (2-AP). The U194C mutation in P9 (black) and the G44C mutation in P3 are also shown. (b) Structure of GAAA tetraloop (orange/red) and 11 nt receptor complex (*1hr2*)⁴⁶, rendered with PyMOL (Delano Scientific). The adenosine replaced by 2AP in the labeled ribozyme is shown in red. Interacting bases in the receptor are shown in green and cyan.

Figure 2. Folding equilibrium of the *Azoarcus* ribozyme by steady-state fluorescence
(a) Emission spectra of the fluorescent wild-type ribozyme at 37 °C, with excitation at 310 nm.
(b) Relative fluorescence intensity (F) at 370 nm in wild-type (black) and P3 mutant (blue) ribozyme *versus* Mg^{2+} concentration. Data were fit to a three-state model as described in Methods. Parameters for statistical weights for wild type: $C_I = 0.29$, $m = 5.0$; $C_N = 0.53$, $n = 7.1$; P3: $C_I = 0.31$, $m = 5.2$; $C_N = 0.58$, $n = 6.2$. Uncertainty in fitted parameters is $\pm 20\%$. As shown in Figure 3, the corresponding transition midpoints for the wild type are: $C_{m,I} = 0.27 \pm 0.04$ mM, $n_{H,I} = 5 \pm 2$; $C_{m,N} = 1.6 \pm 0.2$ mM, $n_{H,N} = 1.7 \pm 0.3$; P3: $C_{m,I} = 0.31 \pm 0.01$ mM, $n_{H,I} = 5 \pm 1$; $C_{m,N} = 15 \pm 2$ mM, $n_{H,I} = 1.0 \pm 0.2$.

Figure 3. Comparison of folding monitored by fluorescence and biochemical probes
(a) Fraction of unfolded (U), intermediate (I_C), and native (N) ribozyme predicted by three-state fits to fluorescence intensity as a function of Mg^{2+} concentration as in Figure 2b. **(b)** Fraction native RNA (f_N) from fluorescence experiments (black line), hydroxyl radical cleavage (red circles, nt 62-64, from Ref. 10) and activity at 32 °C (blue diamonds, data from Ref. 12). Data were fit to the Hill equation (see Table 1).

Figure 4. Folding kinetics by stopped-flow fluorescence

The relative change in 2-AP fluorescence in 15 mM MgCl_2 at 37 °C for (a) wild-type and (b) mutant P3 (G44C) ribozymes (see Materials and Methods). Observed rate constants: wild-type; $k_3 = 2.2 \pm 0.1 \text{ s}^{-1}$, $A_3 = 0.6 \pm 0.2$; $k_4 = 0.23 \pm 0.01 \text{ s}^{-1}$, $A_4 = 0.4 \pm 0.2$; P3 mutant; $k_3 = 11.8 \pm 0.3 \text{ s}^{-1}$, $A_3 = 0.49 \pm 0.01$; $k_4 = 2.24 \pm 0.03 \text{ s}^{-1}$, $A_4 = 0.51 \pm 0.01$. Residuals of the curve fit are shown in red. Insets: Change in fluorescence over 500 ms. Wild-type; $k_1 = 106 \pm 14 \text{ s}^{-1}$, $A_1 = 0.8 \pm 0.1$; $k_2 = 34 \pm 1 \text{ s}^{-1}$, $A_2 = 0.4 \pm 0.2$; $k_3 = 1 \pm 0.5 \text{ s}^{-1}$, $A_3 = 0.6 \pm 0.1$; P3 mutant: $k_1 = 115 \pm 4 \text{ s}^{-1}$, $k_2 = 5 \pm 1 \text{ s}^{-1}$; $k_3 = 0.010 \pm 0.007 \text{ s}^{-1}$. Reported are the mean and standard deviation of three independent trials.

Figure 5. Temperature-dependence of folding kinetics

(a) Folding kinetics at various temperatures (20 - 50 °C) collected over a 500 ms time window. Trajectories were normalized to the minimum and maximum intensity within this time interval. (b) Arrhenius plot for folding of the *Azorarcus* ribozyme. The observed rate constants were averaged over three independent trials. Symbols: red circles, k_1 , $E_{a,1} = 16$ kcal/mol; orange triangles, k_2 , $E_{a,2} = 35$ kcal/mol; green squares k_3 , $E_{a,3} = 19$ kcal/mol; blue diamonds, k_4 , $E_{a,4} = 9$ kcal/mol.

Figure 6. Magnesium-dependence of folding kinetics

Observed folding rate constants (37 °C) in 0.38 to 15 mM MgCl₂ (final). Traces were averaged over three independent trials. Red circles, k_1 , $m_f^\ddagger = 0.3$ kcal/mol; orange triangles, k_2 , $m_f^\ddagger = 0.4$ kcal/mol; green squares, k_3 , $m_f^\ddagger = 0.6$ kcal/mol, $m_u^\ddagger = -0.6$ kcal/mol; blue diamonds, k_4 , $m_f^\ddagger = 0.6$ kcal/mol. The data were fit to $k_{\text{obs}} \approx k_f \exp(b_f + m_f^\ddagger \ln C)$ or $k_3 \approx k_f + k_u \exp(b_f + m_f^\ddagger \ln C) + \exp(b_u + m_u^\ddagger \ln C)$, which assumes a linear change in $\Delta G^\ddagger$ with $\ln C$.

Figure 7. Tertiary folding kinetics of nicked ribozyme by hydroxyl radical footprinting

Folding kinetics of IΔP9•OP9G ribozyme at 37 °C in 15 mM MgCl₂ were measured by time-resolved footprinting (see Methods). (a) Relative saturation of backbone protection (Y) versus folding time for nt 87 (triangles) and nt 88 (circles). Data were scaled to the unfolded and fully-folded RNA and fit to a bi-exponential rate equation (see Methods). (b) Saturation of nt 49 as in (a); arbitrary Y scale shows fluctuations in the relative cleavage of these residues over time. (c) Residues participating in slow refolding steps are colored as in the key. All of these residues except nt 62 are also 40-60% protected in the first 10 ms ($k_1 \geq 100 \text{ s}^{-1}$). Regions that could not be reliably analyzed are shown in gray in the schematics.

Figure 8. Model of ribozyme folding pathways

Based on the data presented here and elsewhere ^{9;10;11;12}, the initial ensemble of secondary structures (U) collapses into native-like (I_C) and non-native intermediates (I's) when the concentration of Mg²⁺ is raised above 0.3 mM. In 15 mM MgCl₂ at 37 °C, native-like intermediates form the native tertiary structure at $\geq 100 \text{ s}^{-1}$. In the nicked IΔP9•OP9G ribozyme, non-native intermediates with P3 mispaired (gold cylinder) require reorganization of the ribozyme core (1 and 0.1 s⁻¹). For simplicity we assume these slow steps occur in parallel, but the data could also be explained by sequential refolding events along the slow pathway.

Scheme I

Table 1Folding equilibria of the *Azoarcus* ribozyme from different experimental techniques.

Technique	U $\rightarrow$ I _C		I _C $\rightarrow$ N	
	C _I (mM)	n _H	C _N (mM)	n _H
SAXS ^a	0.34 $\pm$ 0.01	2.7 $\pm$ 0.01	—	—
RNase T1 ^b	0.2 - 1.1	1.2 - 12.6	—	—
Fluorescence ^c	0.27 $\pm$ 0.04	5 $\pm$ 2	1.6 $\pm$ 0.2	1.6 $\pm$ 0.3
Fe(II)EDTA ^d	—	—	0.7 - 1.8	1.2 - 2.4
Activity ^b	—	—	1.97 $\pm$ 0.06	4.5 $\pm$ 0.5

^aData from Ref. 12 collected at 32 °C.^bData from Ref. 12 collected at 50 °C. Reported are the mean and standard deviation of three independent trials.^cFraction of U, I and N states at 37 °C were used to obtain the parameters for the U $\rightarrow$ I and I $\rightarrow$ N transitions, respectively.^dData from Ref. 10 collected at 50 °C.