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Probing the Folding Landscape of the *Tetrahymena* Ribozyme: Commitment to Form the Native Conformation is Late in the Folding Pathway

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Large, structured RNAs traverse folding landscapes in which intermediates and long-lived misfolded states are common. To obtain a comprehensive description of the folding landscape for a structured RNA, it is necessary to understand the connections between productive folding pathways and pathways to these misfolded states. The *Tetrahymena* group I ribozyme partitions between folding to the native state and to a long-lived misfolded conformation. Here, we show that the observed rate constant for commitment to fold to the native or misfolded states is 1.9 min^{-1} (37 °C, 10 mM Mg^{2+}), the same within error as the rate constant for overall folding to the native state. Thus, the commitment to alternative folding pathways is made late in the folding process, concomitant with or after the rate-limiting step for overall folding. The ribozyme forms much of its tertiary structure significantly faster than it reaches this commitment point and the tertiary structure is expected to be stable, suggesting that the commitment to fold along pathways to the native or misfolded states is made from a partially structured intermediate. These results allow the misfolded conformation to be incorporated into a folding framework that reconciles previous data and gives quantitative information about the energetic topology of the folding landscape for this RNA.

Keywords: folding landscape; group I intron; misfolding; ribozyme; RNA folding

A central goal of RNA folding studies is to obtain a quantitative description of the energetic landscape through which a structured RNA folds to its active form. In principle, this would provide a complete account of the process by which the RNA reaches the native structure from a vast ensemble of disordered states. This description would include all intermediate structures formed transiently, their relative free energies, and their rates of interconversion. Such a quantitative description of the energetic connections between structural forms would allow insight into the nature of the folding steps that connect the intermediates to each other and to the native and unfolded states.

We and others have focused on describing the folding of the *Tetrahymena* group I RNA upon addition of Mg^{2+} under defined conditions *in vitro*, with the aim of quantitatively describing a portion of the energetic landscape[1–15] (for recent reviews, see Brion & Westhof[16] and Treiber & Williamson[17]). This RNA, and the ribozyme derived from it and used herein (Figure 1(a)), fold in the presence of Mg^{2+} in a series of intermediates that contain progressively more tertiary structure.[1,7] Simple folding models were initially proposed, in which the intermediates are ''on-pathway'', connecting the folded and unfolded states (Figure 1(b)). However, it was subsequently shown that at least some intermediates represent misfolded structures, which by definition must unfold partially or completely to fold productively.[5,6,8,9] The rate-limiting

Abbreviations used: DMS, dimethyl sulfate; Mops, 3-(*N*-morpholino)propanesulfonic acid; P, the oligonucleotide product CCCUCU; S, the oligonucleotide substrate $CCCUCUA_5$; S*, (5′-^{32}P)-labeled S.

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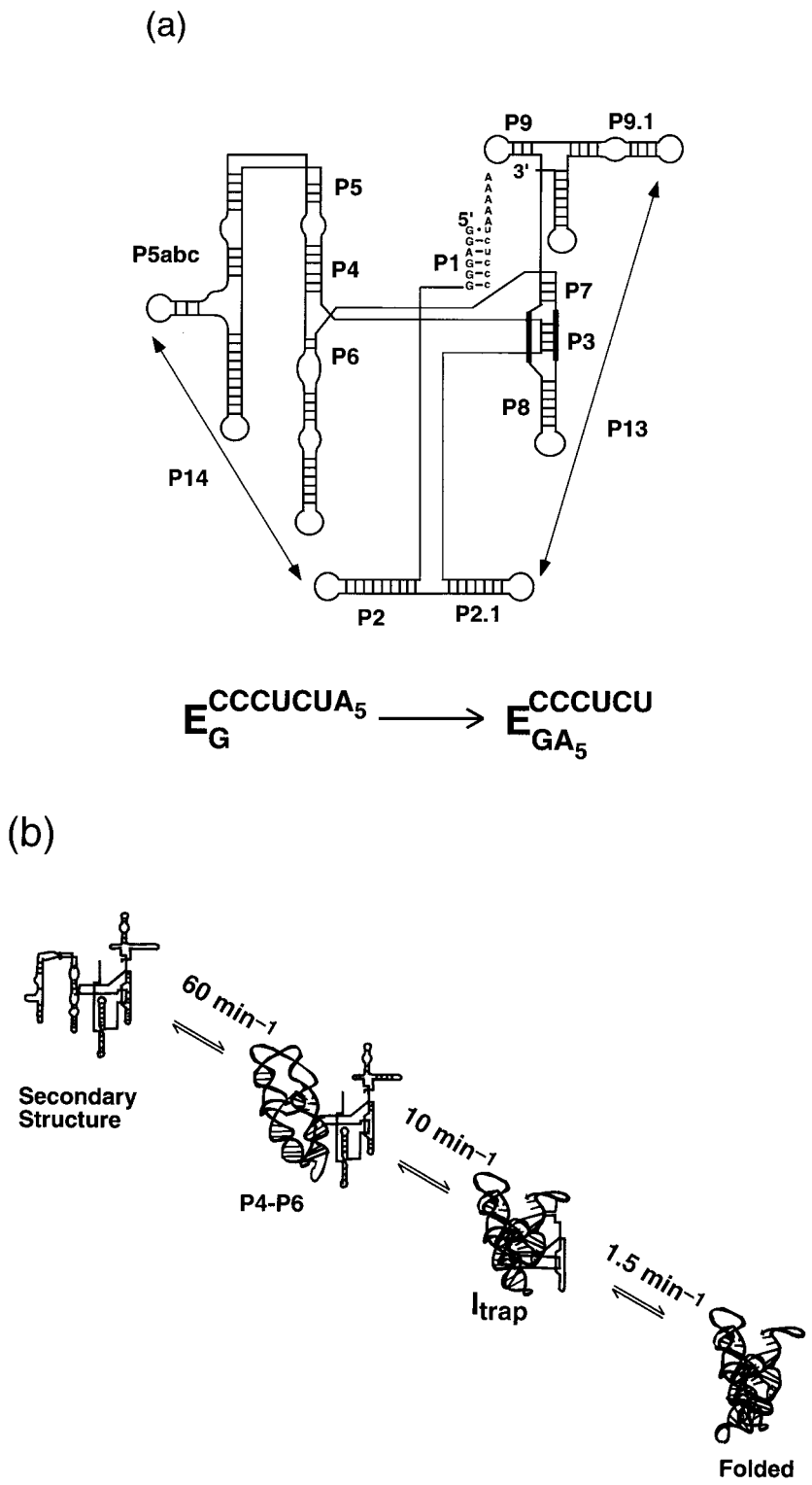
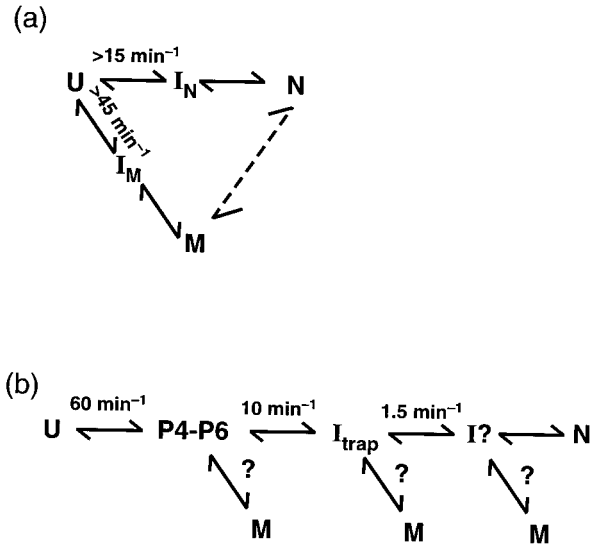


Figure 1. Secondary structure and tertiary folding of the *Tetrahymena* ribozyme. (a) The secondary structure is shown in a representation that reflects the arrangement of the tertiary domains P4-P6 and P3-P8.[34,35] The RNA strands that pair to form the alternative secondary structure Alt P3 are shown with a thicker line.[6,14] The long-range tertiary contacts P13 and P14 are indicated with arrows.[35] The oligonucleotide cleavage reaction is schematically depicted below the secondary structure. (b) A simple model for tertiary folding of the ribozyme (37 °C, pH 7, 10 mM Mg^{2+}, 20 mM Na^{+}).[1,7,11] Portions of the ribozyme with stable tertiary structure are shown as ribbons.

step for overall folding has been suggested to involve partial unfolding of a late intermediate (I_{trap} in Figure 1(b)) that is largely structured and compact,[5,8,10,12,15] suggesting that this intermediate is a transient misfolded species. Additionally, it was shown under standard *in vitro* conditions that 75 % of the population forms long-lived misfolded species not shown in Figure 1(b) and is then stable for at least an hour.†[11]

The simplest expectation is that this long-lived misfolded form(s) lies off of the productive folding pathway(s). However, the connections of this or any other misfolded state with productive folding pathways have not been defined. That is, when do the pathways to the native and misfolded structures diverge? Such knowledge is necessary to place a misfolded intermediate within a quantitative framework for overall folding.

It would be possible for the misfolded and native states to lie along distinct pathways that separate early in folding (Scheme 1(a)), as has been suggested generally for RNA folding from calculated rates of biopolymer condensation.[5,18] Bolstering this view, RNA secondary structure can form much faster than overall folding of large RNAs,[19] and misfolded structures can contain incorrect secondary structures.[6,20,21] Thus, pathways to correct and incorrect structures may diverge early in folding due to the formation of correct or incorrect secondary structures. Alternatively, the misfolded species could represent diversions away from productive pathways late in folding (Scheme 1(b)).[9,10,17] Such late decision points might be favored for RNA because partially structured intermediates can provide a template for formation and stabilization of additional native or non-native interactions.[10,17] No experimental data exist to distinguish these models for any RNA.



Scheme 1.

These two models give contrasting predictions for the point in folding of the ribozyme at which a commitment is made to fold correctly or incorrectly. The model in Scheme 1(a) predicts that as folding begins, each molecule will begin to fold either along the pathway leading to the native state or along the pathway leading to the misfolded state and will rapidly populate intermediates along the pathways. From this early point, the molecule is committed to folding correctly or incorrectly. Thus, Scheme 1(a) predicts that the commitment between pathways will be made faster than intermediates are formed, at least 60 min^{-1}.‡[7] In contrast, the model in Scheme 1(b) predicts a late commitment, after the formation of folding intermediates. In both models, the misfolded states can re-fold to the native state either by unfolding back to the commitment points or through more direct routes (broken lines in Scheme 1(a) and not shown in Scheme 1(b) for clarity).

To probe when the commitment is made to fold to the native or long-lived misfolded states, it was first necessary to determine whether the observed misfolding represents formation of a discrete family of structures or a myriad of unrelated forms. If the latter held, there would be no expectation of a discrete commitment point. We first use site-directed mutagenesis and quantitative kinetic analysis to show that the ribozyme folds largely to a discrete family of misfolded structures. We then

† For simplicity, standard folding conditions are defined throughout as 37 °C, pH 7.0, 10 mM Mg^{2+}, 20 mM Na^{+}.[1,9,11] Although some folding parameters are sensitive to changes in conditions,[1,11] the fraction of the ribozyme that forms long-lived misfolded species is generally insensitive to conditions. Although there is a temperature dependence to this fraction (see Figure 3), a large fraction misfolds from 0 °C to at least 50 °C.[11] Further, changes in monovalent ion identity and concentration, Mg^{2+} concentration, and urea concentration have little or no effect on the fraction that misfolds, at least under conditions such that misfolding is sufficiently long-lived that it is detectable[11] (R.R. & D.H., unpublished results). With high temperature, low Mg^{2+}, high Na^{+}, or high urea concentration the native state is rapidly reached from pre-formed misfolded ribozyme (R.R. & D.H., unpublished results), precluding determination of the fraction of ribozyme that initially misfolds under these conditions.

‡ Structure within P4-P6 is observed to form with a single rate constant of 60 min^{-1},[7] suggesting that essentially the entire population folds quickly to intermediates in which P4-P6 is formed. The observed rate constant for formation of this intermediate, 60 min^{-1}, would be equal to the sum of the rate constants for formation of intermediates along the two pathways in Scheme 1(a).[25] The ratio of the rate constants is determined by the fraction observed to fold to the native state (~0.25).[11] Thus, the transitions from the unfolded ensemble to the folding intermediates in Scheme 1(a) are given rate constants of $\geqslant$45 min^{-1} along the pathway to the misfolded state and $\geqslant$15 min^{-1} along the native pathway. These values are lower limits for the rate constants for formation of intermediates because there may be additional intermediates that were not detected.[7]

address when the commitment is made to fold correctly or incorrectly. The results indicate that the commitment is made late in folding, at a time after much of the structure has formed.

A discrete misfolded conformation

The fraction of the ribozyme population that reaches the native state without stably misfolding was determined by monitoring the onset of enzymatic activity.[11] As a molecule reaches the native state, it quickly cleaves one molecule of oligonucleotide substrate (S), then slowly releases the shortened oligonucleotide product (P) before it can perform another round of cleavage. Thus, in the presence of a small excess of S, a burst of P formation is observed followed by a slower steady-state rate of P formation.[22] The magnitude of the burst, relative to an equivalent reaction in which the ribozyme is pre-folded at 50 °C before the addition of S,[22] gives the fraction of the population that reaches the native state without forming the long-lived misfolded state. The wild-type ribozyme was previously observed to give a burst of 0.27 mol P per mol ribozyme under standard folding conditions.[11] Activity of the remaining 73% was restored by a subsequent 50 °C incubation, ruling out RNA degradation as the source of the low initial activity.

These previous results do not establish whether the ribozyme forms a single family of misfolded conformations or a myriad of unrelated misfolded species. In previous native gel analyses, multiple slowly migrating species were observed, suggesting the presence of multiple misfolded forms for the pre-rRNA[2,5,6] and the ribozyme.[14] However, native polyacrylamide gels are known to slow physical steps and conformational changes in RNA,[23,24] raising the possibility that the gel prevents the interconversion of related species that exchange rapidly in solution. If the exchange in solution were faster than subsequent folding steps, a family of related conformers would behave kinetically as a single species and could give a discrete commitment point in folding, while nevertheless giving multiple bands on a native gel. Supporting the idea of a single family of misfolded species, a variant ribozyme in which the alternative secondary structure Alt P3 is predicted to be destabilized (U273A) has been shown to largely avoid stable misfolding, at least in the presence of low concentrations of Mg^{2+}.[14] This lack of misfolding suggests that a large fraction of the wild-type population forms misfolded species that are related at least by the presence of Alt P3.

To determine whether a large fraction of the ribozyme population also folds to misfolded species in which Alt P3 is formed under the conditions of our experiments (37 °C, pH 6.9, 10 mM Mg^{2+}), folding of the U273A variant was compared with that of the wild-type. In three independent determinations, the U273A variant gave a burst magnitude of 1.04(±0.08) mol P per mol ribozyme relative to a control in which the ribozyme variant was pre-folded at 50 °C (data not shown), much larger than the 0.27 mol P per mol ribozyme burst for the wild-type. The pre-folded U273A ribozyme gave a burst of 0.85(± 0.03) mol P per mol ribozyme based on spectrophotometric concentration determinations of substrate and ribozyme (data not shown), the same within error as the wild-type.

The burst magnitude of 1.04(±0.08) for the U273A variant relative to the pre-folded control indicates that under these conditions it avoids stable misfolding within the limit of detection, suggesting that all detectable misfolded forms for the wild-type contain Alt P3. This result is consistent with a simple model in which the observed misfolding for the wild-type ribozyme results from formation of a family of conformers that rapidly interconvert and so behave as a single species kinetically. It remains possible that multiple families of conformers are populated that contain Alt P3 but do not interconvert.

To determine whether the populated misfolded forms behave as a single species kinetically, conversion of the wild-type ribozyme from the misfolded to native species was followed. If the ribozyme formed multiple misfolded species that were not in rapid equilibrium relative to their conversion to the native state, the observed native state formation from these misfolded species would most simply be expected to give multiple rate constants. In contrast, if the misfolded species were in rapid equilibrium with each other, a single rate constant would be expected.[25]

Formation of the native species was followed for this and subsequent experiments by measuring enzymatic activity with a trace amount of ^{32}P-labeled S (S*) instead of excess S (Figure 2(a)). The input S* partitions between binding to the native and misfolded ribozyme but is cleaved only by the native. The amplitude of the initial burst of cleavage gives the fraction in the native form and does not depend on ribozyme or S* concentration, increasing precision relative to the use of excess S†. Here, the slow phase represents release of S*

† The magnitude of the initial burst of S* cleavage is equal to the fraction of native ribozyme provided that the native and misfolded ribozyme bind S with the same rate constant. Similar rate constants for binding of the two forms are expected because duplex formation with the internal guide sequence limits the rate of S binding to the native form.[22,38,39] In side-by-side comparisons, experiments with trace S* and excess S gave values for the fraction native that differed by less than 20% of the values (data not shown). However, the value obtained by adding trace S* was reproducibly lower than that obtained with excess S, most simply explained by a model in which the misfolded ribozyme binds S slightly faster than the native does. This could arise from a small difference in the local conformation, electrostatic environment, or an increase in the accessibility of the internal guide sequence in the misfolded conformation.

from the misfolded ribozyme and subsequent partitioning between binding to the native ribozyme or re-binding to the misfolded.†

To follow conversion of the misfolded species to the native, the ribozyme was initially folded at 25 °C, giving ~90 % misfolded ribozyme (Figure 2(b)). Conversion to the native form was then followed at 37 °C (Figure 2(c)). The ribozyme slowly converted to the native state with $k_{obs} = 1.4 \times 10^{-3}$ min^{-1} ($t_{1/2} \approx$ eight hours). The observation of a single rate constant is consistent with the presence of a single family of rapidly interconverting misfolded forms.

To further test for the presence of multiple, non-interconverting misfolded species, conversion to the native state was measured under a range of solution conditions. If there were multiple, non-interconverting misfolded species that coincidentally gave similar or identical rate constants under the conditions of Figure 2(c), changes in solution conditions would most simply be expected to have differential effects on conversion of these misfolded species to the native state. However, varying the Mg^{2+} concentration from 1 mM to 10 mM decreased the rate constant for conversion to the native state by 300-fold (Figure 2(d)) but gave a single rate constant in all cases, supporting the presence of a single family of misfolded species. At concentrations of Mg^{2+} above 10 mM, data were consistent with a single rate constant that continued to decrease with increasing Mg^{2+} concentration, but reactions were not followed to completion because of background hydrolysis of the ribozyme on the timescale of days. A single rate constant with a constant value was observed for conversion to native across a range of temperatures for initial formation of misfolded (0-25 °C; data not shown), suggesting formation of the same misfolded species.

These experiments also give information on the properties of the long-lived misfolded species (M) and allow comparison with other folding intermediates. The folding transition from M to the native state is strongly inhibited by Mg^{2+}, suggesting that substantial unfolding of M occurs in the folding transition. In contrast, the rate constant for formation of native ribozyme from the folding intermediate I_{trap} is independent of Mg^{2+} concentration (Figure 2d, 5-100 mM; data from Russell & Herschlag[11]). The transition from I_{trap} to the native state is 1000-fold faster than the transition from the long-lived misfolded state under standard conditions (Figure 2(d)). Although I_{trap} is thought to be a kinetically trapped species that must partially unfold to form the native ribozyme,[8] it has very different properties from M and converts much more readily to the native state under standard conditions.

The commitment point is late in folding

As the observed misfolding results in a single family of misfolded conformers, it is possible that there is a reasonably discrete commitment point between pathways to the native and misfolded states. The commitment point is defined here as follows: at some point in folding the RNA reaches an ensemble of conformations from which two alternative folding events can occur. Within this ensemble, each molecule has the same probability as other molecules of folding to the native or misfolded states; the commitment has not yet been made. Upon completion of one of these alternative events, however, each molecule becomes much more likely to proceed to either the native conformation or the misfolded conformation. The folding ''events'' may be complex processes involving both breakage and formation of contacts, and the commitment is made upon completion of the first essentially irreversible step of the transition. The conformation(s) from which these alternative folding events occur is referred to herein as the commitment point.

To determine whether there is a discrete commitment point between pathways to the native and misfolded states and, if so, to place it in the folding framework, we measured the folding time required to proceed past the commitment point. Observation of a single rate constant for proceeding past the commitment point (k_{commit}) would suggest that the commitment between pathways can be approximated as a discrete commitment point, and determination of the magnitude of k_{commit} would allow the commitment point to be placed in the folding pathway.

The rate constant for commitment to a pathway to the native or misfolded states was measured by folding under one set of conditions for varying amounts of time, then shifting to another set of conditions that gives a different amount of native ribozyme. The fraction of the ribozyme population that has not yet committed to a pathway at the time of the change in conditions will produce the amount of native ribozyme characteristic of the new conditions. Determining the size of the fraction that has not yet committed as a function of time gives the rate constant for commitment, k_{commit}.

† The following results suggest that the slower phase of cleavage activity represents release of S* from the misfolded ribozyme and re-partitioning of S* between the native and misfolded ribozyme. Adding excess unlabeled S (10 μM) after allowing the initial S* to bind to the ribozyme eliminated the slow phase without affecting the initial burst of S* cleavage (data not shown). Additionally, as conversion to the native state proceeded, the rate constant for the slow phase increased proportionally to the magnitude of the burst, from 0.002 min^{-1} with a burst magnitude of 10 % of the input S*, to 0.02 min^{-1} with a burst magnitude of ~80 % (data not shown). This increase is expected from the model above because an increase in the fraction of native ribozyme increases the probability of S* binding to the native ribozyme after release from the misfolded rather than rebinding the misfolded ribozyme.

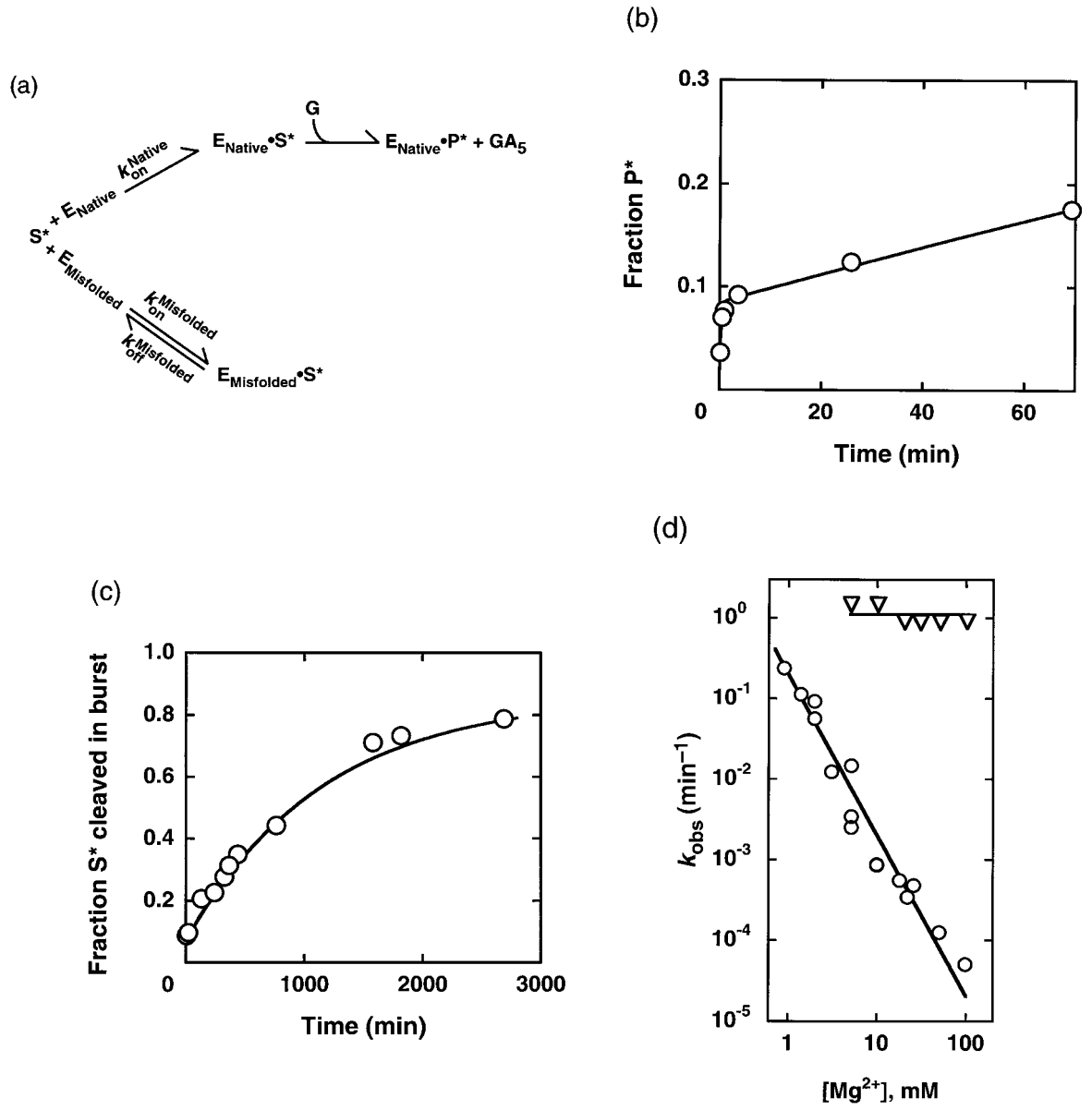


Figure 2 (*legend opposite*)

Temperature was the condition changed in the experiment because we had found previously that 27 % of the ribozyme population folds to the native state without stably misfolding at 37 °C, but only 12 % does so at 25 °C.[11] To measure k_{commit} at 37 °C, folding was initiated by adding Mg^{2+} at 37 °C (Figure 3(a)). After various amounts of time at 37 °C (t_1), the temperature was rapidly changed by dilution to 25 °C. Folding was then allowed to proceed completely to the native and misfolded states at 25 °C ($t_2 \approx 20$ minutes), and the fraction that folded to the native state was determined by enzymatic activity as above.

We found that the fraction of ribozyme that folded to the native state increased with time t_1 at 37 °C (Figure 3(b)). The dependence of the increase in native ribozyme on t_1 was well-described by a single exponential and gave a value for k_{commit} of 1.9(±0.5) min^{-1}. The magnitude of k_{commit} is consistent with Scheme 1(b) and inconsistent with Scheme 1(a). This is because Scheme 1(a) predicts a commitment to alternative pathways with $k_{obs} \geqslant 60$ min^{-1}, faster than formation of the earliest known folding intermediate.[7]

The rate constant of 1.9(±0.5) min^{-1} is the same within error as the rate constant for overall folding. Thus, the commitment between pathways to the native and misfolded states is made late in folding, concomitant with or after the rate-limiting step for overall folding. The value of k_{commit} was the same within error over a range of Mg^{2+} concentrations (6-100 mM; data not shown), suggesting that the late commitment is a general feature of this region of the folding landscape. The results suggest a

Figure 2. Conversion of misfolded ribozyme to the native state. (a) Measurement of the fraction of ribozyme in the native state. A trace concentration of ^{32}P-labeled oligonucleotide substrate (S*) is added to the ribozyme (E) and partitions for binding between the native (E_{native}) and misfolded ($E_{misfolded}$) populations. The fraction of S* that binds to E_{native} is cleaved quickly, much faster than it is released from E_{native}, whereas the fraction that binds to $E_{misfolded}$ is not cleaved until it is released from $E_{misfolded}$ and then can bind E_{native} or again bind $E_{misfolded}$ (see the footnote to page 843). The fraction of S* cleaved quickly is essentially equal to the fraction of ribozyme in the native state (see the footnote to page 842). (b) Monitoring the extent of correct ribozyme folding. After folding 1 μM E for 15 minutes at 25 °C, addition of S* and 1 mM G gave a cleavage burst of magnitude 0.085(±0.003) with k=3.2(±0.5) min^{-1}, followed by a slower phase of S* cleavage (k=0.0015(±0.0001) min^{-1}). (c) Conversion to the native state was followed at 37 °C, 50 mM Na·Mops (pH 6.9), 10 mM Mg^{2+}, 1 μM E. The fraction native (i.e. magnitude of cleavage burst) reached a value indistinguishable from the fraction native after a 50 °C pre-incubation (~0.85; data not shown) previously shown to give maximal activity.[22] Four independent determinations gave a rate constant for native state formation of 1.4(±0.2)×10^{-3} min^{-1}. The observed rate constant was independent of ribozyme concentration from 0.2-2 μM (data not shown), suggesting that conversion to the native state involves a unimolecular rearrangement. (d) Conversion of the ribozyme to the native state from the folding intermediate I_{trap} (▽) or from the stable misfolded state (○). The rate constant for conversion of I_{trap} to the native state was determined by following the initial formation of native ribozyme using excess S (5-100 mM Mg^{2+}, data from Russell & Herschlag;[11] with <5 mM Mg^{2+} the entire population is observed to form the native state with a single rate constant similar to that for the transition from the misfolded to native state, precluding determination of the rate constant for the transition from I_{trap} to native). The line through the data describing conversion from misfolded represents a model in which two Mg^{2+} ions must be released prior to the transition state. For (c) and (d), the fraction in the native form was determined as shown in (b) by removing an aliquot at each time-point and monitoring the burst of product formation at 25 °C. (The Mg^{2+} concentration was increased when necessary to ensure insignificant formation of the native state from the misfolded during the assay.) In initial experiments, a complete 25 °C time-course of S* cleavage was performed for each time of conversion at 37 °C. In subsequent experiments, including the reaction shown in (c), for each time of conversion a single point at one minute of S* cleavage was used to measure the fraction in the native state. At this time-point, the burst is >95% complete, and there is only a negligible contribution from the slow phase (<2%). For all experiments in this work, the L-21 *Sca*I form of the ribozyme was used, and was prepared as described.[11] Reactions were performed in 50 mM Na·Mops (pH 7.0) at 25 °C, and the specified Mg^{2+} concentration, and cleavage of S* was followed by denaturing 20% polyacrylamide gel electrophoresis as described.[11,36]

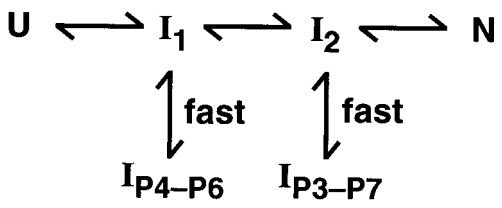
simple model describing the late commitment point in which a late and highly structured folding intermediate, I_{trap} (see Figure 1(b) and below), is not yet committed to a pathway to the native or misfolded states (Figure 3(c)).

Relationship to previous results: a unified folding model

Consideration of previous results,[1,9–11,26] in addition to those herein, leads to a specific model for ribozyme folding with a late commitment point (Figure 4). Previous data suggest that the commitment is made from an intermediate that forms after I_{trap} rather than from I_{trap} itself, as shown in Figure 4 and outlined below. It was previously shown that in the presence of bound S, 40 % of the ribozyme that folds correctly does so much faster than the rest (≥60 min^{-1} compared with ~1 min^{-1}).[26] The rate constant of ~1 min^{-1} for the remainder is the same as that without bound S, suggesting that this slow fraction folds through I_{trap}.[1,11] The fast-folding fraction must avoid I_{trap}, otherwise it could not fold fast, yet the overall fraction that folds correctly is the same with or without bound S.[11,26] This suggests that the fast and slow pathways proceed through a common intermediate from which a folding commitment is made (Figure 4; $I_{commitment}$). This intermediate is placed after I_{trap}, because I_{trap} is formed significantly faster than overall folding (~10 min^{-1})[7] but the commitment to fold correctly or incorrectly is reached more slowly (1.9 min^{-1}, Figure 3(b)).

The model in Figure 4 can reconcile previous data that appear to be inconsistent with results here.[9,10] This work and previous work[11] show that folding of the ribozyme proceeds in two kinetic phases under standard *in vitro* conditions (see footnote (single dagger symbol) on page 841), giving two rate constants that differ by 1000-fold. However, it was previously reported that folding of the ribozyme gave a single observed rate constant under similar conditions.[9,10] This rate constant was suggested to decrease with increasing Mg^{2+} concentration, leading to a model that included a late commitment point between pathways to native and misfolded species (Scheme 2)†. In this model, a kinetically trapped species, termed $I_{P3\text{-}P7}$, is in rapid equilibrium with a late intermediate on a productive pathway (I_2).[10,17] (Note that the rapid equilibrium condition requires only that the late intermediate I_2 essentially always fold to $I_{P3\text{-}P7}$ rather than folding to native (N); it does not require that escape from $I_{P3\text{-}P7}$ is fast.)[25] Increasing stability of the trapped intermediate $I_{P3\text{-}P7}$ by increasing Mg^{2+} concentration then is predicted to decrease the observed rate constant for N for-

† The specific model represented by Scheme 2 was obtained from the description of the ribozyme folding pathway proposed by Rook *et al.*[10] and the more general RNA folding model described by Treiber & Williamson,[17] which incorporated the data of Rook *et al.*[9,10]



Scheme 2.

mation but in all cases give a single rate constant as observed.[10]

To determine the origin of this experimental difference, the observation of one or two kinetic phases in folding, we re-analyzed the previous data[9,10] (Supplementary Material). Although a single rate constant for folding was reported,[10] the fit of a single exponential to the folding data gave a positive *y*-intercept of ~0.2, suggesting that 20% of the population folded with a larger rate constant than that obtained from the fit. The fit also gave a final folded fraction of ~0.6, suggesting that 40% of the population folded with a smaller rate constant than that obtained from the fit. We found that fitting the data by two exponential processes instead of one gives a good fit with the initial and ending values for the fraction of native ribozyme fixed at 0 and 1, respectively. Further, this fit gives results roughly consistent with our data and with other previous results.[1] A fast phase is observed, giving a rate constant of 0.7 min^{-1}, within twofold of the faster rate constants previously observed.[1,11] The slow phase gives a rate constant of 0.02 min^{-1}. Small differences in the identities and concentrations of monovalent cations between our experiments and those of Rook *et al.*[9,10] may account for the 20-fold difference in the rate constant for the slow phase relative to the results here (0.001 min^{-1} *versus* 0.02 min^{-1}), as the rate constant for this transition is extremely sensitive to cations (Figure 2(c) and R.R. & D.H., unpublished results; the earlier work was performed with 25 mM $Tris^+$ and 10 mM Na^+, compared with 20 mM Na^+ here). As described above (Figure 4), we suggest that the fast phase represents formation of native ribozyme from I_{trap}, and the slow phase represents formation of N from M that has formed in the initial phase of folding.

Reconciliation of the data of Rook *et al.*[9,10] with others[1,11] allows a unification of models (Figure 4). This model contains the same species as that of Treiber & Williamson[17] but includes transitions that give two kinetic phases of folding. The intermediate labeled I_{trap} here is the same as that labeled $I_{P4\text{-}P6}$ by Williamson and colleagues, observed by Zarrinkar & Williamson[1] to form the native state with a rate constant of 0.6 min^{-1}. The intermediate labeled M here is the same as that labeled $I_{P3\text{-}P7}$ by Williamson and colleagues, observed to rearrange slowly to the native structure.[10] It is this transition, from M to N, that is inhibited by Mg^{2+}, whereas the transition from I_{trap} to N is unaffected by Mg^{2+} concentration (5-100 mM Mg^{2+}; Figure 2(d)). A central feature of

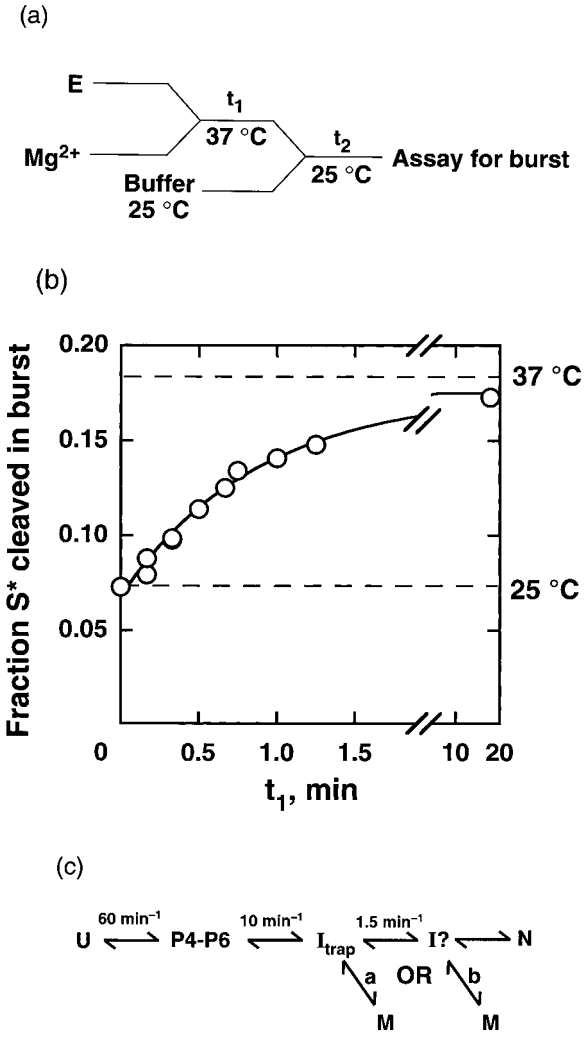


Figure 3. The commitment to fold correctly or incorrectly is made late in folding. (a) Folding was initiated by the addition of 10 mM Mg^{2+} to the ribozyme (1 μM) at 37 °C. Folding proceeded at 37 °C for various times t_1, and aliquots were then diluted fivefold into a 10 mM Mg^{2+} buffer solution at 25 °C. After folding was completed at 25 °C ($t_2 \approx 20$ minutes), the fraction native was determined as described for Figure 2. (b) A late commitment. Four independent determinations gave a rate constant of 1.9(±0.5) min^{-1} for commitment to folding to the native or misfolded states. (c) A model for the late commitment to alternative pathways. The late intermediate I_{trap} may partition between the alternative pathways in the rate-limiting step for overall folding (arrow a), in which case the observed rate constant for folding of 1.5 min^{-1} represents the sum of the rate constants for decay of I_{trap} along the two pathways. The results are also consistent with a model in which an intermediate that is formed from I_{trap} with a rate constant of 1.5 min^{-1} partitions between pathways to the native and misfolded states (arrow b).

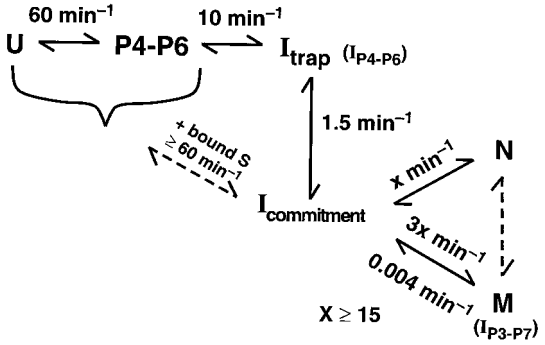


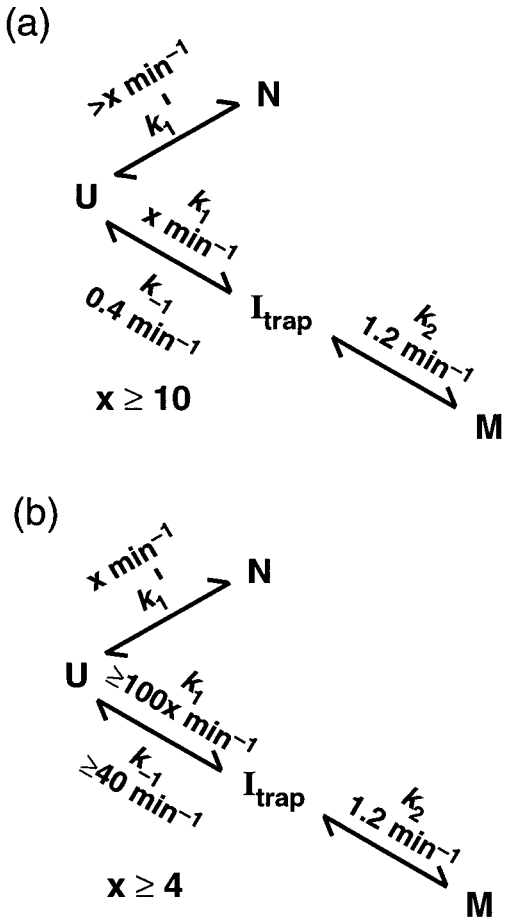
Figure 4. Ribozyme folding model. Under standard folding conditions (37 °C, pH 7, 10 mM Mg^{2+}, 20 mM Na^{+}), essentially the entire population folds through the intermediate I_{trap}. From this intermediate the commitment to fold correctly or incorrectly has not yet been made. I_{trap} is proposed to rearrange in the rate-limiting step for overall folding to an additional intermediate, $I_{commitment}$. This intermediate then rapidly folds to the native and misfolded states (N and M, respectively). The ratio of the rate constants from $I_{commitment}$ to N and M is known from the fraction of ribozyme that folds to N without first folding to M (0.27),[11] giving a ratio of ~1:3. The misfolded ribozyme then slowly converts to the native state with $k_{obs} \approx 10^{-3}$ min^{-1}. Additional experiments suggest that this conversion requires partial unfolding (Figure 2(c) and unpublished results), leading to the model shown here in which the misfolded ribozyme re-forms $I_{commitment}$, from which it can again fold correctly or incorrectly. It is also possible that additional pathways exist for conversion between the native and misfolded states (broken line between N and M). The model includes an additional pathway (diagonal broken line) that bypasses I_{trap} but proceeds through $I_{commitment}$. This pathway is proposed based on experiments showing that in the presence of bound S a fraction of the ribozyme folds fast (>60 min^{-1}) but gives the same fraction of native ribozyme as the slow-folding fraction.[26] This fast folding is not observed in the absence of S (<1 %), presumably because the unpaired internal guide sequence of the ribozyme facilitates formation of I_{trap} in the absence of bound S.[6,37] The observation of fast folding establishes the lower limit of 60 min^{-1} for the decay of $I_{commitment}$ in the presence of S (15 min^{-1} for formation of N from $I_{commitment}$ and 45 min^{-1} for the formation of M), because this step must be at least as fast as the observed overall folding rate constant of >60 min^{-1}. The observation that the overall fraction that folds to the native state is unaffected by the presence of bound S indicates that the ratio of these rate constants is unaffected by S, but the magnitudes of the rate constants may be affected by S. We present the simplest model, in which $I_{commitment}$ gives the same rate constants to the native and misfolded forms with or without bound S.

the general model proposed by Treiber & Williamson,[17] the late commitment point between pathways to the native state and a kinetically trapped intermediate state, is supported experimentally by results herein and is preserved in the model.

Consideration of alternative mechanisms: commitment from structured or unstructured intermediates?

The results here show that the commitment to fold correctly or incorrectly is made with approximately the same rate constant as overall folding. This is counter to the general proposal that pathways to misfolded structures diverge early in folding due to the formation of alternative secondary structures (Scheme 1(a)), because previous results have established that structured intermediates are formed at least 30-fold faster than the commitment to alternative pathways is made (60 min^{-1} *versus* 2 min^{-1}).[1,7,15]

However, these results leave open the possibility that although the commitment is made late in time, it is nevertheless made from an unfolded structure. It can be postulated that the folding transition from I_{trap} to N requires complete unfolding of I_{trap}, again giving an unfolded structure that serves as the commitment point between pathways, either folding to N or returning to I_{trap} (Scheme 3). To consider this model fully, we divide



Scheme 3.

it here into two kinetic classes. In the first class, unfolding of I_{trap} to U is rate-limiting for the transition to N (Scheme 3(a)), while refolding of U to N is rate-limiting in the second class (Scheme 3(b)).

The two classes of the model have several features in common. First, both include the same species and connections between them. As Mg^{2+} is added to initiate folding, U can fold to N or I_{trap}. Once formed, I_{trap} can either unfold to again give U or continue folding to M. Also, in both classes the transition from U to I_{trap} is at least 10 min^{-1} to explain the observed rapid formation of structure in essentially the entire population.[7] It is likely that additional intermediates are formed between U and I_{trap} with even larger rate constants;[7] however, the presence or absence of these intermediates does not affect the analysis of the model, so the additional intermediates are not shown for simplicity. In both versions, I_{trap} is shown as an intermediate on the pathway to M. This is consistent with recent dimethyl sulfate (DMS) protection experiments, which suggest that Alt P3 is formed in both I_{trap} and M (R.R. & D.H., unpublished results). However, the analysis and conclusions are conceptually the same whether I_{trap} is postulated to be on the pathway to the misfolded form, on the pathway to the native form, or an off-pathway intermediate along neither pathway.

The first version of the model (Scheme 3(a)) postulates that, once formed, I_{trap} undergoes a rate-limiting unfolding step back to U, which then rapidly folds to N. This model successfully predicts that if formed, I_{trap} would slowly partition between forming N and M, yet the divergence of pathways would be from the unfolded ensemble. However, the model fails, because it predicts that I_{trap} would never accumulate, so the opportunity for I_{trap} to partition between N and M would not arise. This is because, in order for unfolding to be rate-limiting for the transition from I_{trap} to N, U must form N faster than it returns to I_{trap} ($k_1' > k_1$), so that essentially every time U is formed it folds to N. If this were true, U would fold to N at the start of folding, avoiding I_{trap} formation. However, the fraction of U that avoids I_{trap} to reach N directly is <1 %;[11] i.e. there is no detectable burst of N under these conditions. Thus, k_1 is at least 100-fold larger than k_1', ruling out all models in which the rate constants for formation of N and I_{trap} from U are in the same range as each other, because such models predict an initial burst of N formation from U. Only one class of models remains possible (Scheme 3(b)), with I_{trap} formed from U at least 100-fold faster than N is formed from U ($k_1/k_1' \geqslant 100$, so that $k_1 \gg k_1'$). In addition to this constraint, Scheme 3(b) has unfavorable unfolding of I_{trap} to U ($k_1 > k_{-1}$) because I_{trap} accumulates during folding,[1,7] indicating that its formation from U is thermodynamically favored.

The model in Scheme 3(b) is highly unlikely because it requires unfolding of I_{trap} (k_{-1}) to be very fast. This requirement can be deduced in the following way. The observed rate constant for formation of N from I_{trap}, k_{obs} of 1.5 min^{-1} ([11]; Figure 2(c)), represents the sum of the rate constants for decay of I_{trap} to N ($k_{I_{trap} \rightarrow N}$) and M (equation (1)).[25] Additionally, the fraction

$$k_{obs} = k_{I_{trap} \rightarrow N} + k_2 \quad (1)$$

of ribozyme that is observed to form N rather than M (0.27)[11] represents the probability of forming N from I_{trap} and is therefore a ratio of rate constants (equation (2)). The simultaneous solution

$$\text{Fraction N} = \frac{k_{I_{trap} \rightarrow N}}{k_{I_{trap} \rightarrow N} + k_2} \quad (2)$$

of equations (1) and (2), using the values of k_{obs} (1.5 min^{-1}) and fraction N (0.27), gives $k_{I_{trap} \rightarrow N}$ equal to 0.4 min^{-1}.

Under the unfavorable equilibrium and rapid equilibrium conditions of Scheme 3(b) ($k_1 > k_{-1}$ and $k_1 \gg k_1'$), the overall rate constant $k_{I_{trap} \rightarrow N}$ is a function of the individual rate constants in equation (3). This allows determination of a value for k_{-1}

$$k_{I_{trap} \rightarrow N} = k_{-1}\left(\frac{k_1'}{k_1}\right) \quad (3)$$

the rate constant for I_{trap} unfolding in Scheme 3(b). The overall rate constant $k_{I_{trap} \rightarrow N}$ represents the rate constant for formation of U from I_{trap} (k_{-1}) multiplied by the probability of forming N from U rather than re-forming I_{trap} (k_1'/k_1). The probability of forming N from U instead of returning to I_{trap} is $\leqslant 0.01$ ($= k_1'/k_1$), based on the absence of an observed burst of N formation from U as described above.[11] This value and the value of $k_{I_{trap} \rightarrow N}$ of 0.4 min^{-1} obtained above give $k_{-1} \geqslant 40$ min^{-1}, according to equation (3).

Rapid unfolding of I_{trap}, with a rate constant of $\geqslant 40$ min^{-1}, is unlikely because I_{trap} has been shown to be a highly structured intermediate in which the independently folding domain P4-P6 is formed[1,7] and long-range contacts including the loop-loop contacts P13 and P14 appear to be formed.[7] Even if only the P4-P6 domain were formed, I_{trap} would be unlikely to unfold fast enough to allow viability of Scheme 3(b). Solution radical protection studies give an equilibrium constant of ~100 for formation of the isolated P4-P6 domain ($K_{P4\text{-}P6}$).[27] The measured rate constant for P4-P6 formation of 60 min^{-1} under these conditions[7,28,29] divided by $K_{P4\text{-}P6}$ gives a calculated value of 0.6 min^{-1} for unfolding of P4-P6, >60-fold less than required by Scheme 3(b).

For the P4-P6 domain to unfold with a rate constant of $\geqslant 40$ min^{-1} in the context of I_{trap}, it would appear that the domain must be much less stable than it is in isolation. One could imagine that P4-P6 is formed improperly in I_{trap}, facilitating its unfolding relative to the properly folded, isolated P4-P6 domain. This appears unlikely, however, as the patterns of protection from solution radicals for the intradomain contacts of P4-P6 are similar in

I_{trap} and the isolated domain.[30,31] Further, P4-P6 acquires stable tertiary structure with similar rate constants in the presence and absence of the rest of the ribozyme, suggesting that similar structures are formed.[7,28,29] Additional contacts of the P4-P6 domain with other portions of the ribozyme could, in principle, destabilize the properly folded P4-P6 in the context of I_{trap} relative to P4-P6 in isolation. However, the simplest expectation is that additional contacts, notably the formation of the P14 base-pairs between P5c and P2, would stabilize P4-P6 in I_{trap} relative to the isolated domain, as these additional contacts provide substantial stabilization to P4-P6 in the native structure.[27]

The preceding discussion strongly suggests that I_{trap} does not unfold completely during its transition to N. We therefore conclude that the commitment point between pathways to the native and misfolded structures is likely to be a structured intermediate. Nevertheless, it is quite possible that some unfolding of I_{trap} does occur prior to the divergence of pathways to the native and misfolded states,[8] in which case the intermediate at the commitment point ($I_{commitment}$ in Figure 4) could be less structured than I_{trap}.

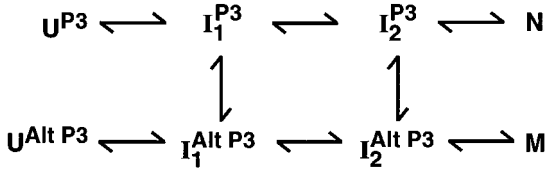
Decision points and commitment points in folding

Although the commitment point characterized here is late in folding, there are likely to be multiple ''decision'' points or branches between alternative pathways to the native and misfolded states, both early and late (Scheme 4). For practical reasons, we emphasize commitment points herein. This is because each decision point will be undetectable unless it commits molecules to fold *via* alternative pathways.

A decision point will be a commitment point in folding if interconversion of subsequent intermediates between the pathways is much slower than the completion of folding. For example, consider a folding population that begins folding with the alternative secondary structure Alt P3 formed ($U^{Alt\ P3}$ in Scheme 4), and subsequently folds to the intermediate and decision point $I_1^{Alt\ P3}$, which will serve as a commitment point in folding if it can fold to either $I_2^{Alt\ P3}$ or I_1^{P3} as shown, and interconversion of the subsequent intermediates, $I_2^{Alt\ P3}$ and I_2^{P3}, is slow relative to the completion of folding (transitions from $I_2^{Alt\ P3}$ to M and from I_2^{P3} to N).† This is because slow interconversion of $I_2^{Alt\ P3}$ and I_2^{P3} relative to the completion of folding means that a molecule at the I_2 stage of folding is essentially fixed in one pathway or the other. Whether or not a decision point serves as a commitment point is thus a quantitative question, dependent on the rate of interconversion between pathways relative to the rates of subsequent folding steps.

The folding transitions that give conversion between the pathways may be quite different from those that give completion of folding and therefore may have different dependencies on conditions such as temperature or Mg^{2+} concentration. Changing folding conditions, then, may perturb the landscape such that the position of the commitment point in folding is changed. Indeed, additional experiments indicate that a late intermediate is committed to misfold at 15 °C but not at 37 °C (R.R. & D.H., unpublished results), suggesting that the commitment point is earlier at the lower temperature.

The position of the commitment point could also be dependent on the starting position in the landscape, as this could influence which decision points are encountered or avoided on the pathways to the folded state(s). Consistent with this idea, there is evidence that the commitment point between pathways to the native and misfolded states can be early in folding. Data from the Woodson laboratory[14] and herein show that the variant U273A, in which P3 is stabilized relative to the alternative duplex Alt P3, avoids misfolding. Recent DMS protection experiments suggest that P3 is formed in this variant in the absence of Mg^{2+}, whereas Alt P3 is formed in the wild-type (R.R. & D.H., unpublished results). Thus, for this ribozyme it appears that an early decision, the formation of P3 instead of Alt P3 in the starting ensemble, determines the outcome of folding. This is illustrated in Scheme 4 by initiating folding from U^{P3} rather than from $U^{Alt\ P3}$. Supporting this early commitment, initiating folding of the wild-type ribozyme from solution conditions that give initial P3 formation also allows mostly correct folding (R.R., X. Zhuang, H. Babcock, S. Chu, & D.H., unpublished results). The late decision point, $I_{commitment}$ in Figure 4 (and conceptually $I_1^{Alt\ P3}$ or $I_2^{Alt\ P3}$ in Scheme 4) is presumably avoided in each of the above cases due to the initial formation of P3. In contrast, for the wild-type ribozyme under standard conditions Alt P3 is formed initially, but the correct and incorrect secondary structures are



Scheme 4.

† For $I^{Alt\ P31}$ to serve as a commitment point it is also required that I_1^{P3} fold to I_2^{P3} much faster than it returns to $I_1^{Alt\ P3}$. If, instead, I_1^{P3} rapidly reformed $I_1^{Alt\ P3}$, these two intermediates ($I_1^{Alt\ P3}$ and I_1^{P3}) would be in rapid equilibrium relative to the completion of folding and would together form a commitment point.

apparently able to exchange even late in the folding pathway.

Conclusions

We have found that the folding landscape for the *Tetrahymena* ribozyme contains a commitment point late in folding between pathways to the native state and a long-lived misfolded state. Essentially the entire population folds through the intermediate I_{trap}, but then a significant fraction of the population continues to fold to the native state while the rest forms a long-lived misfolded form. Although an incorrect secondary structure is formed early in folding, it is not until late that the ribozyme is committed to fold correctly or incorrectly. Additional tertiary contacts that form late may be responsible for stabilizing the correct and incorrect structures, preventing their exchange. It is also possible that more subtle effects such as small changes in positioning of domains with respect to each other give increased stability late in folding, hindering exchange of late intermediates on the pathways to the native and misfolded states.

The two observed kinetic phases of folding, representing transition from I_{trap} to native and transition from the long-lived misfolded form to native, give rate constants that differ by 1000-fold under standard conditions. The transitions also give different dependencies on Mg^{2+} concentration, as the transition from I_{trap} to native is unaffected by Mg^{2+} concentration whereas the transition from misfolded to native is strongly inhibited by Mg^{2+}. This provides further evidence that these species are distinct and provides some initial characterization of differences in their properties. Further work will be required to understand the structural differences between these two folding intermediates and the different folding transitions that they undergo to form the native state.

Finally, it is interesting to consider what regions of RNA folding landscapes are likely to contain commitment points between alternative folding pathways. The rapid and stable formation of secondary structure for RNA leads to the expectation of early commitment points because the folding pathway traversed may be dependent on which base-pairs form early.[5,18] However, early formation of alternative base-pairs may not give an early commitment point if the alternative duplex is not sufficiently long-lived to persist through folding, as exchange of the correct and incorrect duplexes can allow interconversion between the pathways. Indeed, the Alt P3 duplex is formed early, yet the commitment point is late in folding. The four base-pair Alt P3 duplex is predicted to dissociate as an intermolecular duplex with a half-life of <1 ms at 37 °C.[32,33] Thus, late commitment points may arise because secondary structure formed early is able to exchange during folding until it is fortified by additional structure.

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References

1. Zarrinkar, P. P. & Williamson, J. R. (1994). Kinetic intermediates in RNA folding. *Science,* **265**, 918-924.
2. Emerick, V. L. & Woodson, S. A. (1994). Fingerprinting the folding of a group I precursor RNA. *Proc. Natl Acad. Sci. USA,* **91**, 9675-9679.
3. Downs, W. D. & Cech, T. R. (1996). Kinetic pathway for folding of the *Tetrahymena* ribozyme revealed by three UV-inducible crosslinks. *RNA,* **2**, 718-732.
4. Zarrinkar, P. P. & Williamson, J. R. (1996). The kinetic folding pathway of the *Tetrahymena* ribozyme reveals possible similarities between RNA and protein folding. *Nature Struct. Biol.* **3**, 432-438.
5. Pan, J., Thirumalai, D. & Woodson, S. A. (1997). Folding of RNA involves parallel pathways. *J. Mol. Biol.* **273**, 7-13.
6. Pan, J. & Woodson, S. A. (1998). Folding intermediates of a self-splicing RNA: mispairing of the catalytic core. *J. Mol. Biol.* **280**, 597-609.
7. Sclavi, B., Sullivan, M., Chance, M. R., Brenowitz, M. & Woodson, S. A. (1998). RNA folding at millisecond intervals by synchrotron hydroxyl radical footprinting. *Science,* **279**, 1940-1943.
8. Treiber, D. K., Rook, M. S., Zarrinkar, P. P. & Williamson, J. R. (1998). Kinetic intermediates trapped by native interactions in RNA folding. *Science,* **279**, 1943-1946.
9. Rook, M. S., Treiber, D. K. & Williamson, J. R. (1998). Fast folding mutants of the *Tetrahymena* group I ribozyme reveal a rugged folding energy landscape. *J. Mol. Biol.* **281**, 609-620.
10. Rook, M. S., Treiber, D. K. & Williamson, J. R. (1999). An optimal Mg(2+) concentration for kinetic folding of the *Tetrahymena* ribozyme. *Proc. Natl Acad. Sci. USA,* **96**, 12471-12476.
11. Russell, R. & Herschlag, D. (1999). New pathways in folding of the *Tetrahymena* group I RNA enzyme. *J. Mol. Biol.* **291**, 1155-1167.
12. Pan, J. & Woodson, S. A. (1999). The effect of long-range loop-loop interactions on folding of the *Tetrahymena* self-splicing RNA. *J. Mol. Biol.* **294**, 955-965.
13. Pan, J., Thirumalai, D. & Woodson, S. A. (1999). Magnesium-dependent folding of self-splicing RNA: exploring the link between cooperativity, thermodynamics, and kinetics. *Proc. Natl Acad. Sci. USA,* **96**, 6149-6154.
14. Pan, J., Deras, M. L. & Woodson, S. A. (2000). Fast folding of a ribozyme by stabilizing core interactions: evidence for multiple folding pathways in RNA. *J. Mol. Biol.* **296**, 133-144.
15. Russell, R., Millett, I. S., Doniach, S. & Herschlag, D. (2000). Small angle X-ray scattering reveals a com-

pact intermediate in RNA folding. *Nature Struct. Biol.* **7**, 367-370.
16. Brion, P. & Westhof, E. (1997). Hierarchy and dynamics of RNA folding. *Annu. Rev. Biophys. Biomol. Struct.* **26**, 113-137.
17. Treiber, D. K. & Williamson, J. R. (1999). Exposing the kinetic traps in RNA folding. *Curr. Opin. Struct. Biol.* **9**, 339-345.
18. Thirumalai, D. & Woodson, S. A. (1996). Kinetics of folding of proteins and RNA. *Accts. Chem. Res.* **29**, 433-439.
19. Turner, D. H., Sugimoto, N. & Freier, S. (1990). Thermodynamics and kinetics of base-pairing and of DNA and RNA self-assembly and helix coil transition. *Nucl. Acids,* **16**, 201-207.
20. Wu, M. & Tinoco, I., Jr (1998). RNA folding causes secondary structure rearrangement. *Proc. Natl Acad. Sci. USA,* **95**, 11555-11560.
21. Silverman, S. K., Zheng, M., Wu, M., Tinoco, I., Jr & Cech, T. R. (1999). Quantifying the energetic interplay of RNA tertiary and secondary structure interactions. *RNA,* **5**, 1665-1674.
22. Herschlag, D. & Cech, T. R. (1990). Catalysis of RNA cleavage by the *Tetrahymena thermophila* ribozyme. 1. Kinetic description of the reaction of an RNA substrate complementary to the active site. *Biochemistry,* **29**, 10159-10171.
23. Konarska, M. M. & Sharp, P. A. (1986). Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. *Cell,* **46**, 845-855.
24. Emerick, V. L., Pan, J. & Woodson, S. A. (1996). Analysis of rate-determining conformational changes during self-splicing of the *Tetrahymena* intron. *Biochemistry,* **35**, 13469-13477.
25. Fersht, A. (1999). *Structure and Mechanism In Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, W.H. Freeman and Company, USA.
26. Zhuang, X., Bartley, L. E., Babcock, H. P., Russell, R., Ha, T., Herschlag, D. & Chu, S. (2000). A single molecule study of RNA catalysis and folding. *Science,* **288**, 2048-2051.
27. Ralston, C. Y., He, Q., Brenowitz, M. & Chance, M. R. (2000). Stability and cooperativity of individual tertiary contacts in RNA revealed through chemical denaturation. *Nature Struct. Biol.* **7**, 371-374.
28. Deras, M. L., Brenowitz, M., Ralston, C. Y., Chance, M. R. & Woodson, S. A. (2000). Folding mechanism of the *Tetrahymena* ribozyme P4-P6 domain. *Biochemistry,* **39**, 10975-10985.
29. Silverman, S. K., Deras, M. L., Woodson, S. A., Scaringe, S. A. & Cech, T. R. (2000). Multiple folding pathways for the P4-P6 RNA domain. *Biochemistry,* **39**, 12465-12475.
30. Sclavi, B., Woodson, S., Sullivan, M., Chance, M. R. & Brenowitz, M. (1997). Time-resolved synchrotron X-ray ''footprinting'', a new approach to the study of nucleic acid structure and function: application to protein-DNA interactions and RNA folding. *J. Mol. Biol.* **266**, 144-159.
31. Murphy, F. L. & Cech, T. R. (1993). An independently folding domain of RNA tertiary structure within the *Tetrahymena* ribozyme. *Biochemistry,* **32**, 5291-5300.
32. Serra, M. J. & Turner, D. H. (1995). Predicting thermodynamic properties of RNA. *Methods Enzymol.* **259**, 242-261.
33. Xia, T., SantaLucia, J., Jr, Burkard, M. E., Kierzek, R., Schroeder, S. J., Jiao, X., Cox, C. & Turner, D. H. (1998). Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base-pairs. *Biochemistry,* **37**, 14719-14735.
34. Cech, T. R., Damberger, S. H. & Gutell, R. R. (1994). Representation of the secondary and tertiary structure of group I introns. *Nature Struct. Biol.* **1**, 273-280.
35. Lehnert, V., Jaeger, L., Michel, F. & Westhof, E. (1996). New loop-loop tertiary interactions in self-splicing introns of subgroup IC and ID: a complete 3D model of the *Tetrahymena thermophila* ribozyme. *Chem. Biol.* **3**, 993-1009.
36. Herschlag, D., Eckstein, F. & Cech, T. R. (1993). Contributions of 2′-hydroxyl groups of the RNA substrate to binding and catalysis by the *Tetrahymena* ribozyme. An energetic picture of an active site composed of RNA. *Biochemistry,* **32**, 8299-8311.
37. Woodson, S. A. & Cech, T. R. (1991). Alternative secondary structures in the 5′ exon affect both forward and reverse self-splicing of the *Tetrahymena* intervening sequence RNA. *Biochemistry,* **30**, 2042-2050.
38. Bevilacqua, P. C., Kierzek, R., Johnson, K. A. & Turner, D. H. (1992). Dynamics of ribozyme binding of substrate revealed by fluorescence-detected stopped-flow methods. *Science,* **258**, 1355-1358.
39. Herschlag, D. (1992). Evidence for processivity and two-step binding of the RNA substrate from studies of J1/2 mutants of the *Tetrahymena* ribozyme. *Biochemistry,* **31**, 1386-1399.

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Supplementary Material comprising a Figure showing previous ribozyme folding experiments is available at IDEAL.