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Slow folding kinetics of RNase P RNA

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ABSTRACT

Understanding the folding mechanisms of large, highly structured RNAs is important for understanding how these molecules carry out their function. Although models for the three-dimensional architecture of several large RNAs have been constructed, the process by which these structures are formed is only now beginning to be explored. The kinetic folding pathway of the *Tetrahymena* ribozyme involves multiple intermediates and both Mg^{2+}-dependent and Mg^{2+}-independent steps. To determine whether this general mechanism is representative of folding of other large RNAs, a study of RNase P RNA folding was undertaken. We show, using a kinetic oligonucleotide hybridization assay, that there is at least one slow step on the folding pathway of RNase P RNA, resulting in conformational changes in the P7 helix region on the minute timescale. Although this folding event requires the presence of Mg^{2+}, the slow step itself does not involve Mg^{2+} binding. The P7 and P2 helix regions exhibit distinctly different folding behavior and ion dependence, implying that RNase P folding is likely to be a complex process. Furthermore, there are distinct similarities in the folding of RNase P RNA from both *Bacillus subtilis* and *Escherichia coli*, indicating that the folding pathway may also be conserved along with the final structure. The slow folding kinetics, Mg^{2+}-independence of the rate, and existence of intermediates are basic features of the folding mechanism of the *Tetrahymena* group I intron that are also found in RNase P RNA, suggesting these may be general features of the folding of large RNAs.

Keywords: kinetic oligonucleotide hybridization assay; Mg^{2+}-dependence; ribozymes; RNA folding; subdomains

INTRODUCTION

RNA and RNA structure can play an essential role during almost every step of gene expression and genome replication. Most strikingly, there is evidence that the catalytic component of both the spliceosome and the ribosome may be RNA (Noller, 1993; Madhani & Guthrie, 1994). In addition to these natural functions, in vitro selection methods have produced RNAs with a range of novel ligand binding and catalytic properties (Joyce, 1994). The functional versatility of RNA is made possible by the ability to fold into complex three-dimensional structures, and a complete understanding of RNA function requires answering two questions. First, what are the final structures of active RNAs and second, what is the process by which the active structures are formed? Together, these two questions define an RNA folding problem analogous to the much studied protein folding problem (Pyle & Green, 1995). The suggestion has also been made that information on RNA folding pathways may be of use for the computational prediction of RNA structures (Gultyaev et al., 1995; Konings & Gutell, 1995). Although much progress has been made recently in elucidating the three-dimensional structures of RNA (Pyle & Green, 1995; Shen et al., 1995), insight into the folding mechanisms of large RNAs is only now beginning to emerge (Celander & Cech, 1991; Banerjee et al., 1993; Jaeger et al., 1993; Emerick & Woodson, 1994; Laggerbauer et al., 1994; Zarrinkar & Williamson, 1994, 1996a, 1996b; Banerjee & Turner, 1995; Pan, 1995; Pyle & Green, 1995; Weeks & Cech, 1995, 1996).

We have studied the *Tetrahymena* ribozyme as a model system for RNA folding and have proposed a model for the kinetic folding mechanism of this group I intron (Zarrinkar & Williamson, 1994, 1996a, 1996b). Folding occurs by a complex process involving multiple intermediates and both Mg^{2+}-dependent and Mg^{2+}-independent steps, with the two major subdomains in the structure of this ribozyme forming in a hierarchical manner. Short-range secondary structure elements,

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such as simple hairpins, form rapidly and do not require Mg^{2+}. Several long-range base pairing interactions in each of the subdomains are not present or are unstable in the absence of Mg^{2+}, and their formation/stabilization can be observed upon the addition of Mg^{2+}. The overall rate-limiting step, taking place on the timescale of minutes ($k_{obs} = 0.72\ min^{-1}$), precedes the stable formation of the second subdomain. The slow step, which in itself does not involve binding of Mg^{2+}, is driven forward by subsequent rapid Mg^{2+} binding to a transient intermediate, and can thus only take place in the presence of Mg^{2+}. Although several specific tertiary interactions have been demonstrated to be formed during the slow step, it is unknown which of the microscopic folding events is actually rate limiting. Formation of individual helical elements of the slower folding subdomain is interdependent, and this structural subunit therefore has been proposed to correspond to a kinetic folding unit. One question that arises from these studies is whether the folding mechanism of the *Tetrahymena* ribozyme is exceptional, or whether the features of the pathway are relevant to RNA folding in general.

To address this question, we chose to study folding of the RNA component of ribonuclease P (RNase P) which, like group I introns, is a relatively large, highly structured molecule. RNase P is a ribonucleoprotein enzyme found in all organisms and plays a crucial role in the production of mature tRNA by cleaving nucleotides from the 5′ end of pre-tRNA (Altman et al., 1995). The RNA component of RNase P from many prokaryotes can catalyze this reaction alone, in the absence of protein, whereas the protein component of the enzyme is required for activity in higher organisms (Darr et al., 1992; Altman et al., 1995). Like group I introns, RNase P RNAs from different species contain a core region of highly conserved interactions that is surrounded by additional structural elements that can vary substantially (Darr et al., 1992; Haas et al., 1994). The enzymes from *Escherichia coli* and *Bacillus subtilis* are among the best-studied RNase P molecules, and, for both, the RNA alone is an efficient catalyst. They are of similar size as the *Tetrahymena* ribozyme (close to 400 nt) and their secondary structure has been determined from phylogenetic comparison and biochemical studies (Darr et al., 1992; Haas et al., 1994) (Fig. 1). Models for the three-dimensional architecture of the *E. coli* RNA have been proposed (Harris et al., 1994; Westhof & Altman, 1994), and a detailed kinetic framework for the reaction of *B. subtilis* RNase P RNA with a pre-tRNA substrate is also available (Beebe & Fierke, 1994), providing a very powerful functional assay. Furthermore, an equilibrium study of the Mg^{2+}-induced folding of the *B. subtilis* RNA has provided evidence for the existence of several subdomains of tertiary structure (Pan, 1995). This study also revealed that, although optimal catalytic activity of RNase P RNA requires the presence of high concentrations of monovalent metal ions or Mg^{2+} (Reich et al., 1988; Darr et al., 1992; Brown et al., 1993), stable higher-order structure can be formed at relatively low concentrations of Mg^{2+}, even in the absence of additional monovalent metals.

As in the *Tetrahymena* ribozyme and most other large RNAs, at least two different classes of base pairing interactions are apparent in the secondary structure of RNase P RNA (Fig. 1). First, there are short-range hairpin stem-loop interactions, which are expected to form very rapidly and at low salt concentrations, with no requirement for divalent metals. Second, there are long-range interactions, such as the P2, P4, and P7 helices, where regions of the RNA far apart in the linear sequence fold back on each other. In analogy to our findings in the group I ribozyme, the long-range interactions may require Mg^{2+} for their stable formation, and may form more slowly than short-range interactions.

To begin to elucidate the kinetic folding pathway of RNase P RNA, we have applied a kinetic oligonucleotide hybridization assay. Our results show that folding of the RNAs from both *E. coli* and *B. subtilis* is slow, on the timescale of minutes, and that the slow step results in stabilization of structure in the P7 helix region. The slow folding event requires the presence of Mg^{2+}, but does not in itself involve binding of Mg^{2+}. The P2 helix region showed folding behavior distinct from that of P7 in both RNAs, suggesting the existence of a complex folding pathway with multiple intermediates. The slow timescale of folding and the Mg^{2+}-independence of the slow step, together with the differential behavior of separate parts of the molecule, recapitulate some of the key features of the folding pathway of the *Tetrahymena* group I intron. The folding mechanisms of the two classes of ribozymes may therefore illustrate general features of the folding of large, highly structured RNA molecules.

RESULTS

Kinetic oligonucleotide hybridization assay

To study RNase P folding, we decided to adapt the oligonucleotide hybridization assay used previously in our investigations of group I intron folding (Zarrinkar & Williamson, 1994). The assay takes advantage of the differential accessibility of the RNA to binding by short, complementary DNA probes in the presence and absence of Mg^{2+} and/or other metal ions, and allows monitoring the kinetics of folding of specific regions of the RNA. In general, formation of higher-order structure in large RNAs, such as RNase P and group I introns, requires Mg^{2+} or other divalent metals (Celander & Cech, 1991; Pan et al., 1993; Pyle, 1993), whereas much of the short-range secondary structure can form at very low concentrations of monovalent metal ions. The ad-

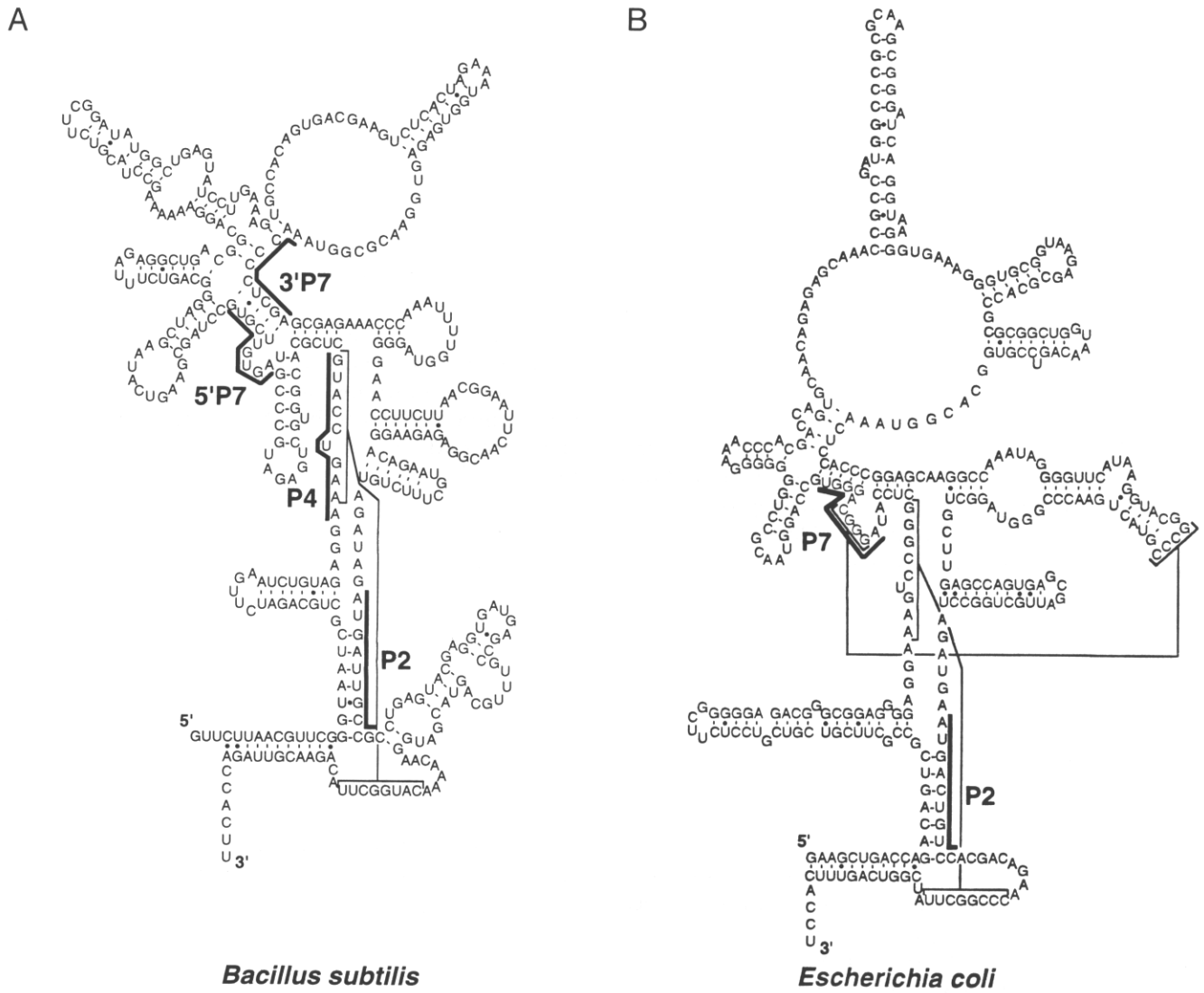

FIGURE 1. Secondary structures of RNase P RNA. Sequences complementary to oligonucleotide probes are indicated by bold lines. Probes are named according to the secondary structure element they target.

dition of Mg^{2+} together with a relatively high concentration of Na^+ or NH_4^+ is therefore expected to allow formation of the native conformation from a partially folded state. In the hybridization assay, higher-order folding is induced by the addition of metal ions, and the fraction folded after increasing incubation times is determined by adding a DNA probe, complementary to a specific sequence in the RNA, together with RNase H. The probe will bind to RNA still unfolded, and RNase H, an enzyme that specifically digests the RNA strand of an RNA:DNA hybrid, will cleave the RNA:probe complex. Probe binding and RNase H cleavage are allowed to proceed for a short time before the reaction is quenched, providing a "snapshot" of progress toward the native state. Separation of cleaved and uncleaved RNA by electrophoresis followed by quantitation of the fraction cleaved after different folding times reveals the time-course of the transition from the unfolded, accessible, to the folded, inaccessible, conformation. By using probes complementary to different parts of the RNA, folding of each region can, in principle, be followed specifically and independently. In practice, a prerequisite for the application of this assay is the existence of sequences in the RNA that display substantial changes in accessibility in the presence and absence of Mg^{2+} and/or moderately high concentrations of monovalent metal ions. A second limitation of the assay is the requirement for RNase H cleavage, which restricts the solution conditions for folding to those in which RNase H is active, and prevents the use of high ion concentrations (>200 mM).

To determine the suitability of the kinetic oligonucleotide hybridization assay for studying the folding of RNase P, we prepared a series of four probes comple-

mentary to different sequences in *B. subtilis* RNase P RNA (Fig. 1A). The targeted nucleotides are involved in long-range base pairing interactions, similar to those successfully studied with this assay in the *Tetrahymena* ribozyme (Zarrinkar & Williamson, 1994), which may be expected to require Mg^{2+} for their formation. The probes were complementary to both strands of the P7 helix, one strand of the P4 helix, or one strand of the P2 helix. P7 participates in a four-helix junction and is part of what appears to be an independently folding subdomain (Pan, 1995). P4 is a long-range interaction proposed to be a component of the active site (Harris & Pace, 1995), and P2 links regions near the 5′ and 3′ ends of the molecule. For each of these sequences, we asked whether the target is accessible to oligonucleotide binding and/or RNase H cleavage if the probe is added simultaneously with Mg^{2+} and a moderately high concentration of NH_4^+, corresponding to the zero time point of a kinetic experiment, and whether it is accessible after the RNA has been equilibrated under folding conditions for 10 min (Fig. 2). We chose to fold the RNA in 100 mM NH_4Cl and 20 mM $MgCl_2$, where RNase H retains full activity in our assay. Although not optimal, these conditions still support RNase P catalytic activity (Tallsjö & Kirsebom, 1993; Kufel & Kirsebom, 1994), and should allow stable formation of higher-order structure (Pan, 1995). In our assay, both strands of P7 showed metal ion-dependent differences in accessibility, with the effect being much more pronounced for the strand closer to the 5′ end of the RNA (using probe 5′P7), indicating that P7 is not stable in the absence of metal ions. P2 also displayed differential accessibility with and without preincubation under folding conditions. P4, however, was completely inaccessible, even when the probe was added together with Mg^{2+} and NH_4^+, suggesting that, although it is a long-range interaction, it is stable under less stringent conditions than other elements of the secondary structure.

The observed changes in the accessibility of the RNA upon folding suggested that RNase P is amenable to kinetic analysis by the oligonucleotide hybridization assay. The changes were substantial for the P2 and 5′P7 probes, and we therefore decided to use these probes to investigate the kinetics of folding. There are several conditions that must be met for the assay to report the actual folding rate in a kinetic experiment (Zarrinkar & Williamson, 1994). First, binding of the probe and RNase H cleavage must be fast compared to folding, so that no additional folding takes place once probe and RNase H are added. Second, RNase H cleavage must be fast compared to dissociation of the RNA:probe complex to ensure that all of the RNA bound by the probe will be cleaved. Third, unfolding of the RNA under native conditions must be slow compared to folding, to prevent cleavage of RNA that had already folded at the time probe and RNase H are added. Finally, high

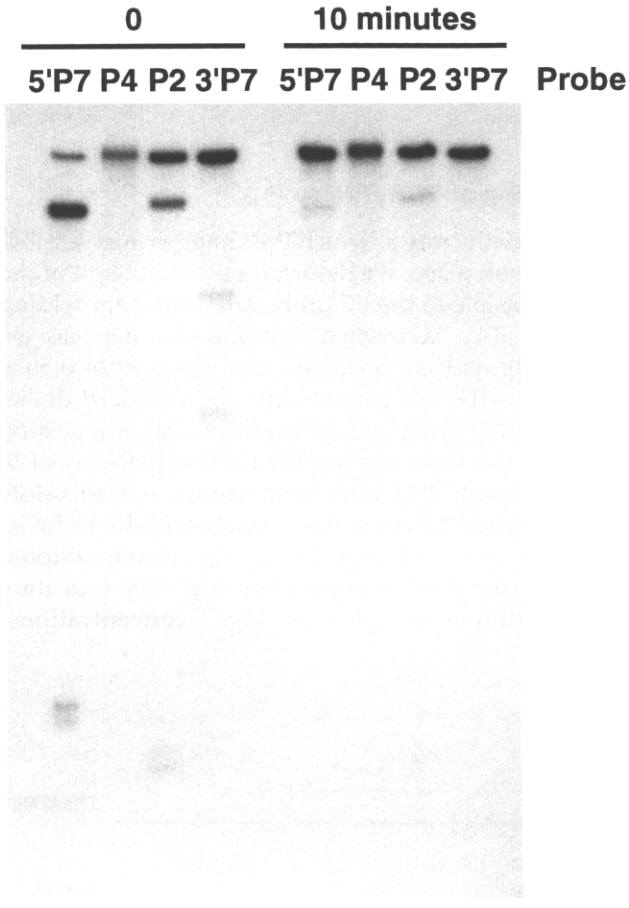

FIGURE 2. Oligonucleotide hybridization screen of *B. subtilis* RNase P RNA. For the zero time point, probes and RNase H were added to the RNA together with folding buffer, and for the 10-min time point, the RNA was first incubated in folding buffer for 10 min, as described in Materials and Methods. Final probe concentrations were 410 μM for 5′P7, 400 μM for P4, 460 μM for P2, and 320 μM for 3′P7.

enough probe concentrations must be used to ensure rapid binding of all accessible RNA. Control experiments to ensure that each of these conditions was met in our standard assay procedure were performed with each probe used in kinetic experiments (data not shown). The controls showed that probe binding and RNase H cleavage were complete in less than 30 s and that less than 10–20% of RNA:probe complex dissociated within 30 s after addition of the probe. When the RNA was allowed to fold in 20 mM $MgCl_2$/100 mM NH_4Cl for 15 min prior to addition of probe and RNase H, less than 20% cleavage was observed after 30 s, indicating that, during the time the RNA is exposed to probe and RNase H, it does not unfold significantly, and that the probe does not induce denaturation of the RNA on this timescale. The concentration of each probe necessary to produce maximal cleavage of the RNA was determined by measuring RNA cleavage at the zero time point, as described above, using a series of probe concentrations. The kinetic oligonucleotide

hybridization assay therefore does accurately measure the rate of folding under the conditions used in the experiments reported here (see the Materials and methods).

Slow folding of P7 in RNase P

To measure the rate at which the changes in accessibility of P7 took place, we determined the fraction of the RNA accessible to the P7 probe after different folding times (Fig. 3A). Accessibility was found to decrease exponentially with an observed rate constant of 0.50 ± 0.13 min^{-1} (the error represents the standard deviation from five independent experiments), suggesting that there is a slow step on the folding pathway of *B. subtilis* RNase P RNA and, furthermore, that this slow step is required for the stable formation of the P7 helix.

We next asked whether the folding event monitored by this probe was dependent on Mg^{2+} by repeating the experiment at different Mg^{2+} concentrations, while keeping the NH_4^+ concentration constant. As the Mg^{2+} concentration was lowered, the fraction of RNA accessible to probe binding and/or RNase H cleavage at equilibrium increased, indicating that the folding transition only took place in the presence of Mg^{2+} (Fig. 3A). However, the observed rate constant of folding was independent of the Mg^{2+} concentration. Therefore, there must be at least two individual steps involved in this folding event: a slow, Mg^{2+}-independent step, the rate of which is measured in our experiments, and a rapid Mg^{2+}-binding step that may allow the Mg^{2+}-independent step to proceed. These observations thus reveal the presence of at least one kinetic intermediate on the folding pathway. The fraction of RNA cleaved at equilibrium at different Mg^{2+} concentrations was used to construct an equilibrium folding curve (Fig. 3B). The midpoint of the transition ($[Mg^{2+}]_{1/2}$ = 3.2 mM) was very close to that reported by monitoring the protection of the RNA from Fe(II)-EDTA induced hydroxyl radical cleavage ($[Mg^{2+}]_{1/2}$ = 2–3 mM) (Pan, 1995), suggesting that the two techniques may report the same folding transition.

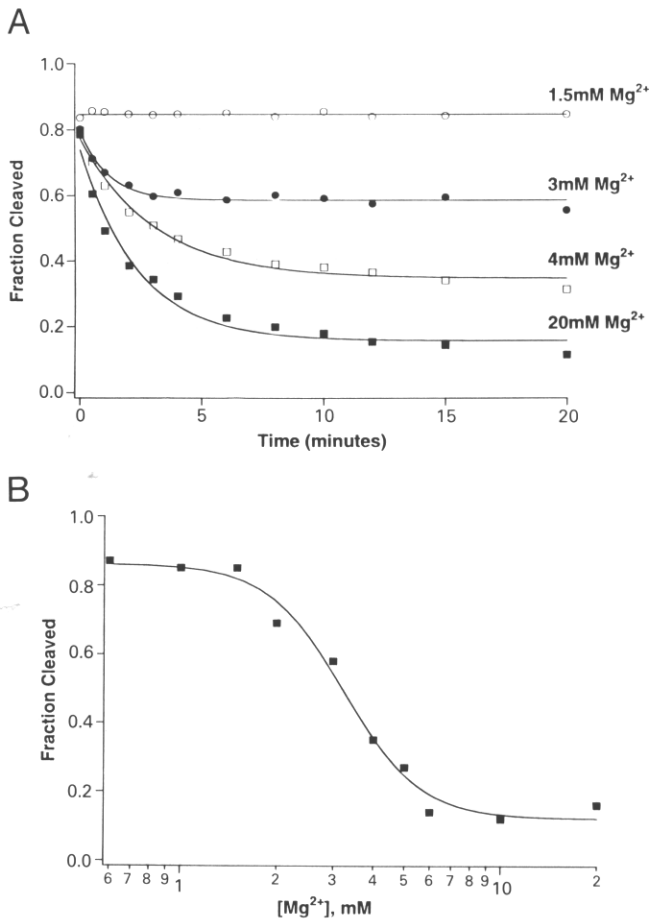

FIGURE 3. Folding of the P7 region in *B. subtilis* RNase P. **A:** Folding kinetics at different Mg^{2+} concentrations. **B:** Equilibrium Mg^{2+} dependence. Each point represents the endpoint of a kinetic experiment as in A, performed at a different Mg^{2+} concentration. Values for $[Mg^{2+}]_{1/2}$ and n were 3.2 mM and 3.6, respectively. The final probe concentration (5′P7) was 70 μM.

Folding behavior of P2

When the folding kinetics of the RNA were measured using a probe targeting the P2 helix, we found behavior very different from that of the P7 region. At 20 mM Mg^{2+}, a substantial fraction of the RNA remained accessible to the probe at equilibrium (Fig. 4). Increasing the Mg^{2+} concentration to 40 mM did not result in any additional protection (data not shown). Conversely, omitting Mg^{2+} altogether, and folding the RNA in the presence of NH_4^+ only, still resulted in significant protection. Only when the NH_4^+ concentration was lowered to 10 mM, in the absence of Mg^{2+}, did the RNA become almost completely accessible to probe binding and/or RNase H cleavage (Fig. 4). Sodium was

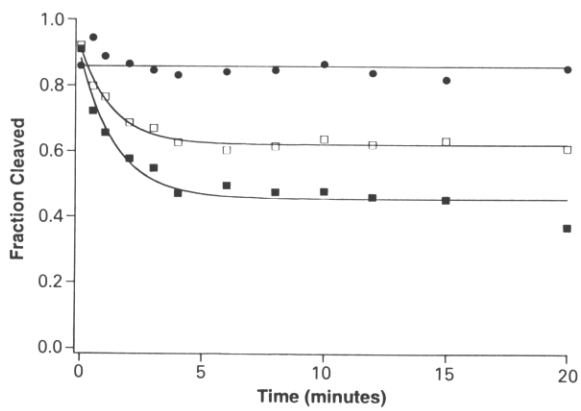

FIGURE 4. Formation of P2 in *B. subtilis* RNase P. Folding conditions were: ■, 20 mM $MgCl_2$/100 mM NH_4Cl; □, 0 mM $MgCl_2$/100 mM NH_4Cl; ●, 0 mM $MgCl_2$/10 mM NH_4Cl. The final probe concentration was 40 μM.

found to be as effective as NH_4^+ in inducing protection of the RNA, and the effect is therefore not specific for NH_4^+ (data not shown). These observations indicate that different subregions of RNase P RNA exhibit distinctly different folding behavior upon the addition of metal ions.

Folding of RNase P RNA from *E. coli*

To determine whether the folding behavior of RNase P is conserved between different species, we repeated the experiments described above with the ribozyme from *E. coli*, again using probes targeting P7 and P2. In *E. coli*, the secondary structure in the P7 region differs from that in *B. subtilis* RNA and nucleotides next to P7, targeted by the P7 probe, are involved directly in another long-range interaction (Fig. 1). The folding rate constant measured with the P7 probe in *E. coli* (Fig. 5A) ($k_{obs} = 0.68 \pm 0.07$ min^{-1}, where the error represents the standard deviation from four independent experiments) was nevertheless very similar to that found for *B. subtilis* RNA. As in *B. subtilis*, the apparent rate constant was independent of the concentration of Mg^{2+}, whereas the extent of folding at equilibrium declined at lower Mg^{2+} concentrations (Fig. 5A). The midpoint of the equilibrium transition ($[Mg^{2+}]_{1/2} = 2.4$ mM in *E. coli*, 3.2 mM in *B. subtilis*) was also similar in the two species (Fig. 5B).

The P2 region was again protected by NH_4^+, even in the absence of Mg^{2+} (Fig. 6). In *E. coli*, however, the protection was almost complete, whereas in *B. subtilis*, only partial protection of this region was observed. Furthermore, there was only a minimal loss of protection upon lowering the NH_4^+ concentration from 100 mM to 10 mM (Fig. 6). Even in the absence of any added metal ions, the P2 region was partially protected by the addition of buffer alone (data not shown). The rate of the folding event monitored by the P2 probe appeared to change at different metal ion concentrations, but the changes were not systematic and not consistent enough to allow accurate quantitation. The data do suggest, however, that this region behaves similarly, in a qualitative fashion, in both species of RNase P RNA examined, and that this behavior is distinctly different from that of the P7 region.

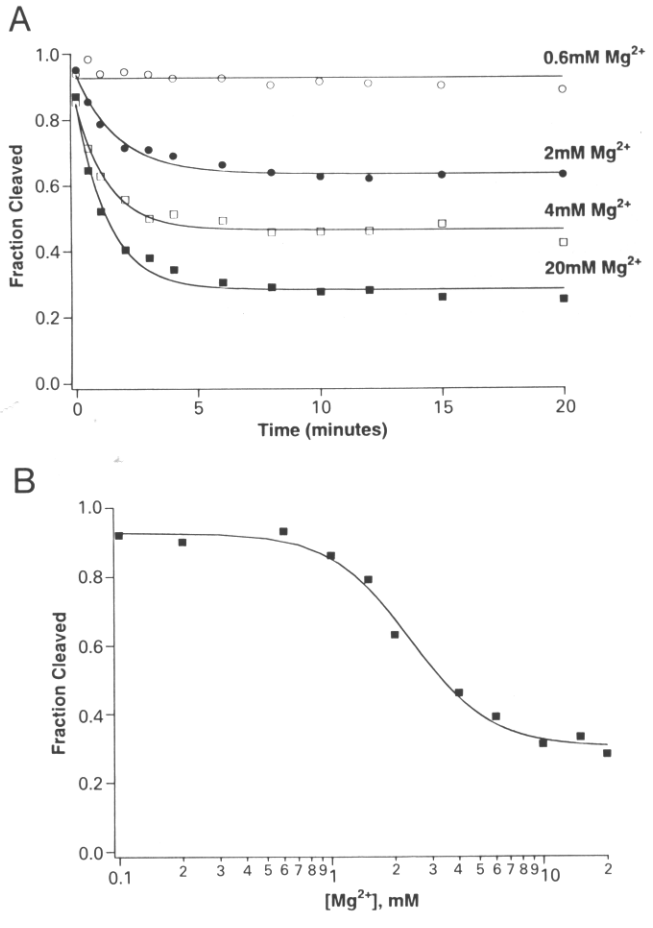

FIGURE 5. Folding of the P7 region in *E. coli* RNase P. **A:** Folding kinetics at different Mg^{2+} concentrations. **B:** Equilibrium Mg^{2+} dependence. Each point represents the endpoint of a kinetic experiment as in A, performed at a different Mg^{2+} concentration. Values for $[Mg^{2+}]_{1/2}$ and n were 2.4 mM and 2.3, respectively. The final probe concentration was 70 μM.

DISCUSSION

We have initiated a study of the kinetic folding mechanism of RNase P RNA. A series of control experiments show that a kinetic oligonucleotide hybridization assay, developed to study folding of the *Tetrahymena* ribozyme, can be applied to obtain information on RNase P folding. An initial screen of several regions of *B. subtilis* RNase P RNA revealed changes in the accessibility of the RNA to binding by short, complementary oligodeoxynucleotides and/or cleavage of the resulting complexes by RNase H upon the addition of metal ions.

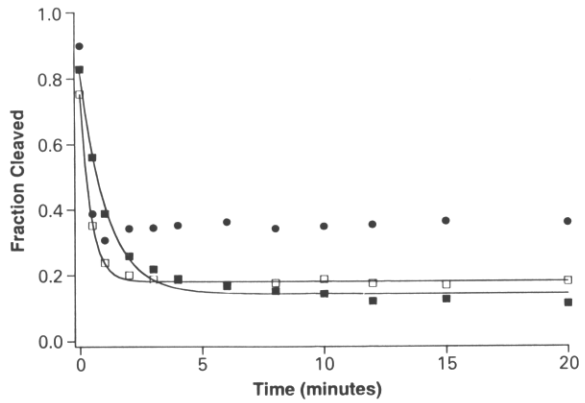

FIGURE 6. Formation of P2 in *E. coli* RNase P. Folding conditions were: ■, 20 mM $MgCl_2$/100 mM NH_4Cl; □, 0 mM $MgCl_2$/100 mM NH_4Cl; ●, 0 mM $MgCl_2$/10 mM NH_4Cl. The final probe concentration was 70 μM.

Slow folding kinetics

The most significant changes were observed for a probe targeting the P7 region of the RNA. When we measured the rate at which these changes occurred, it was found to be slow, taking place on the timescale of minutes ($k_{obs} = 0.50 \pm 0.13$ min^{-1}, Fig. 3A). The observation of a slow step with a probe targeting P7 and adjacent sequences suggests that structure formation in this region either is involved directly in the slow step, or that the rearrangement occurring during the slow step is required for stable structure to form in this section of the RNA. The Mg^{2+} concentration independence of the rate of this slow step shows that it does not involve binding of Mg^{2+}. The existence of at least two steps must be invoked to explain the apparent contradiction of the requirement for Mg^{2+}, but Mg^{2+} independence of the slow step itself, providing evidence for at least one kinetic intermediate on the folding pathway.

A complex folding pathway

The sequences targeted by the four probes used in the initial screen for changes in accessibility each exhibited a different pattern of protection (Fig. 2). The 5′P7 target sequence was almost completely accessible when the probe was added together with metal ions, but became almost completely inaccessible after incubation of the RNA under folding conditions. The 3′ strand of P7, however, showed less dramatic changes in accessibility. This asymmetry observed for P7 is probably explained by the two probes targeting adjacent sequences that are part of different structural elements, in addition to nucleotides forming P7 itself. P7 is one of the helices forming a four-way junction, and the 3′P7 probe is centered on this junction, possibly reporting mainly its formation or stabilization, rather than that of P7 itself. The results suggest that the four-way junction may be marginally stable in the absence of added metal ions, and becomes stabilized upon their addition. The 5′P7 probe, however, does not extend across the junction, but rather also targets nucleotides in a bulge adjacent to P7, and may therefore report predominantly the formation of structure involving this bulged region.

The equilibrium Mg^{2+} concentration dependence of folding detected with the 5′P7 probe is consistent with published results using Fe(II)-EDTA generated hydroxyl radicals as a probe for higher-order structure formation (Pan, 1995). The midpoint of the transition ($[Mg^{2+}]_{1/2} = 3.2$ mM for oligonucleotide hybridization, 2–3 mM for hydroxyl radical footprinting), as well as the number of magnesium ions involved (3–4) was similar in both studies, suggesting that the two techniques may report on the same folding event.

The P4 helix, which is a long-range interaction and is proposed to be part of the active site (Harris & Pace, 1995), is inaccessible even if the probes are added together with metal ions, suggesting either that it is already stable in the absence of added metal, or that it forms extremely rapidly upon the addition of metal ions, much faster than binding of the DNA probe to its target sequence. In the Fe(II)-EDTA footprinting study (Pan, 1995), P4 was accessible in the absence of Mg^{2+}. This apparent discrepancy with our results may be explained if base pairing in P4, which would afford protection from binding by our probe, is stable early during folding and in the absence of Mg^{2+}, but if the higher-order interactions, which afford protection from hydroxyl radical cleavage, do not occur unless Mg^{2+} is present. Protection of the P2 helix, which links sequences near the two ends of the RNA, is not absolutely dependent on Mg^{2+}, and occurs in the presence of moderate concentrations of monovalent ions. This suggests that base pairing in this helix also may be stabilized sufficiently to exclude our probes even in the absence of higher-order structure. Alternatively, under the conditions used for the folding studies reported here, which are less than optimal for catalytic activity, the P2 helix region may be involved in non-native interactions (i.e., misfolding) that may result in partial protection from our probes. The current representation of the RNase P secondary structure (Fig. 1) includes P2, whereas P4 is only indicated indirectly. The apparent greater stability and/or more rapid formation of P4 revealed by our results suggests that an alternative representation, which includes P4 and where P2 is indicated indirectly, may be more appropriate. The variable behavior exhibited by different regions of the RNA in response to metal ions suggests that folding of RNase P does not occur in a single, cooperative transition, but that structure is formed under different conditions in different parts of the RNA, and that the folding mechanism is likely to be complex.

Central features of RNA folding

Although the secondary structure of RNase P RNA from *E. coli* differs in several places from that of *B. subtilis*, including the region surrounding the P7 helix (Fig. 1), the folding behavior of the two regions tested here in both molecules is very similar. There is a slow step involving structure formation in the P7 region in both RNAs, and this slow step, while only taking place in the presence of Mg^{2+}, in itself does not involve binding of Mg^{2+}. The P2 region of both RNAs becomes at least partially inaccessible in the presence of only moderate concentrations of monovalent ions. In spite of the differences in degree, these findings imply at least some conservation of the folding mechanism between these two species of RNase P. Although the extent of conservation remains to be defined, it is consistent with the hypothesis that the final structure and the pro-

cess by which the structure is formed are linked, and do not evolve independently.

A comparison of RNase P folding with the folding pathway of the *Tetrahymena* ribozyme also reveals that several aspects of group I intron folding are conserved in RNase P. In both ribozymes, different regions exhibit distinctly variable patterns of accessibility to complementary oligonucleotide probe binding and/or RNase H cleavage. In the *Tetrahymena* ribozyme, these differences reflect a complex kinetic folding mechanism with multiple steps and intermediates (Zarrinkar & Williamson, 1994, 1996a, 1996b). It seems likely that a similarly complex folding pathway will be revealed for RNase P. The second clear similarity is the existence of a slow step not involving binding of Mg^{2+}, and taking place on the timescale of minutes, on the folding pathway of both RNAs. Both the rate of the slow step, and the concentration of Mg^{2+} required to allow it to proceed, are very similar in RNase P and the *Tetrahymena* group I intron (Zarrinkar & Williamson, 1994). Furthermore, there are reports that folding of a group II intron (Griffin et al., 1995) and another group I intron (Lewin et al., 1995; Weeks & Cech, 1996) also is slow, taking place at timescales similar to those found here and for the *Tetrahymena* ribozyme. Our results from the *Tetrahymena* ribozyme and from RNase P, together with these reports, suggest that slow kinetics and a complex folding pathway including multiple intermediates may be common features of higher-order folding of large RNAs. The Mg^{2+} independence of the rate of the slow step in both RNase P and the group I ribozyme may also indicate that the nature of the rate-limiting step itself could be similar in different RNAs. Furthermore, the recent demonstration that the RNA component of a simple RNP, composed of a group I intron and a specifically bound protein, must form its core structure before protein binding can occur shows that the kinetics of RNA folding can govern the kinetics of RNP assembly (Weeks & Cech, 1996). The present results may therefore be relevant not only to folding of RNAs, but also to assembly of multicomponent RNPs.

The studies described here provide a foundation for future, more detailed investigations of the folding mechanism of RNase P. There are several major questions that need to be addressed. First, to test whether the slow folding step identified here is the overall rate-limiting step during folding, it will be necessary to measure the rate at which the active structure of the RNA is formed by a functional assay. Second, the importance of structural subdomains during folding of RNase P, and their possible hierarchical relationship, needs to be defined in greater detail. In the group I ribozyme, structural subdomains have been correlated with kinetic folding units, and their formation is a hierarchical process (Zarrinkar & Williamson, 1996a). Evidence for distinct subdomains and a hierarchical relationship between them in RNase P (Pan, 1995) may lead to similar observations. Third, what is the nature of the slow folding step? Does it involve formation of interactions that allow structural subdomains to interact, as in the *Tetrahymena* ribozyme (Zarrinkar & Williamson, 1996a, 1996b), or are structural rearrangements within a single subdomain responsible? Finally, it will be of great interest to examine the effect of the RNase P protein on folding. The protein alleviates the need for high ionic strength for optimum catalytic activity, and is believed to stabilize the structure of the RNA as well as facilitate binding of the pre-tRNA substrate (Reich et al., 1988; Altman et al., 1995). It may also, however, influence the kinetics of folding.

In summary, we have provided evidence that some of the central aspects of the folding mechanism of the *Tetrahymena* ribozyme are conserved in RNase P RNA. The folding pathways of these two ribozymes may therefore illustrate general features of higher-order RNA folding.

MATERIALS AND METHODS

Oligonucleotide synthesis

Oligodeoxynucleotide probes were synthesized on a 1 μmol scale on an Applied Biosystems DNA synthesizer, deprotected overnight at 65 °C in 2 mL concentrated ammonium hydroxide, and purified on 20% denaturing polyacrylamide gels. Full-length bands were excised and eluted from the gel overnight into water at 4 °C, followed by desalting on C_{18} Sep-Paks (Waters).

RNase P RNA preparation

Ribozymes were prepared by transcription from plasmids pDW66 (*B. subtilis*) and pDW98 (*E. coli*) linearized with *Dra* I and *Sna*B I (New England Biolabs), respectively. Transcription reactions (100 μL) were performed in 40 mM Tris·HCl, pH 7.5, 2 mM spermidine, 10 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM each ATP, CTP, UTP, 0.1 mM GTP, and 400 μCi of [α-^{32}P] GTP (3,000 Ci/mmol, New England Nuclear) for 3.5 h at 37 °C using 500 units T7 RNA polymerase (New England Biolabs) and 15 μg linearized plasmid template. Full-length transcription products were purified on 6% denaturing polyacrylamide gels and eluted from the gel overnight at 4 °C into buffer containing 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 0.3 M sodium acetate. The RNA was ethanol precipitated and resuspended in 10 mM Tris·HCl, pH 7.5/0.1 mM EDTA. Ribozyme concentrations were determined by Cerenkov counting.

Oligonucleotide hybridization assay

Experiments were performed as described previously (Zarrinkar & Williamson, 1994). RNase P (final concentration 1 nM) in 60 μL of buffer containing 1 mM Tris·HCl, pH 7.5, and 0.01 mM EDTA was annealed by heating to 95 °C for 45 s followed by equilibration at 37 °C for 3 min. Folding was initiated by addition of an equal volume of 2× folding buffer

(1×: 50 mM Tris·HCl, pH 7.5, 100 mM NH_4Cl, 1 mM dithiothreitol) which, unless otherwise noted, also contained $MgCl_2$ to give the desired concentration. Aliquots (10 μL) were taken at the times indicated and added to 10 μL of 1× folding buffer containing oligonucleotide probe and RNase H (United States Biochemical, final concentration 0.1 U/μL) and enough $MgCl_2$ to bring the final concentration to 20 mM. Oligonucleotide binding and RNase H cleavage were allowed to proceed for 30 s before the reaction was quenched with 14 μL of stop solution (90 mM EDTA and marker dyes in 82% formamide). The zero time points were obtained by adding oligonucleotide probe and RNase H in 2× folding buffer and $MgCl_2$ (final concentration 20 mM) to the RNA immediately after annealing in a separate reaction. Products were separated on 6% denaturing polyacrylamide gels and quantitated using a Molecular Dynamics PhosphorImager. The data were fit to single exponentials to obtain values for the observed rate constant and equilibrium endpoint. No change in the folding rate of P7 was observed when the annealing protocol was altered to lengthen heating at 95 °C to 90 s, or the incubation at 37 °C to 10 min. When the RNA from either species was annealed in 0.5 mM $MgCl_2$/100 mM NH_4Cl before addition of sufficient $MgCl_2$ to induce stable formation of P7, a small decrease in the folding rate constant of P7 was observed (k_{obs} ~0.2 min^{-1}). Slow folding is therefore independent of the annealing conditions, although the observed rate constant can vary slightly. To obtain the Mg^{2+} concentration dependence of folding at equilibrium, kinetic experiments were performed in folding buffer containing varying concentrations of Mg^{2+}. During the probe binding/RNase H cleavage step the Mg^{2+} concentration was always adjusted to 20 mM. The equilibrium endpoint from the fit of the data to single exponentials yielded the fraction of RNA folded at equilibrium for each Mg^{2+} concentration. The data were fit to an expression of two-state binding of n Mg^{2+} ions [$f = 1/\{([Mg^{2+}]/[Mg^{2+}]_{1/2})^n + 1\}$], where f is the fraction cleaved at equilibrium, n the number of Mg^{2+} ions bound per RNA molecule, and $[Mg^{2+}]_{1/2}$ the midpoint of the transition.

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