A conserved bulged adenosine in a peripheral duplex of the antigenomic HDV self-cleaving RNA reduces kinetic trapping of inactive conformations

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ABSTRACT

In the ribozyme of hepatitis delta virus antigenomic RNA, two short duplexes, P2 and P2a, stabilize the active self-cleaving structure. However, P2a also promotes kinetic trapping of non-native structures. A bulged adenosine (A14) separates P2a and P2; this bulged A is conserved in clinical isolates of HDV but is unlikely to be physically close to the cleavage site phosphate in the ribozyme structure. Removing the bulge did not significantly slow the rate of cleavage but slowed the conversion of inactive to active conformations. In the absence of the bulged A, inactive conformations required higher urea concentrations or higher temperatures to be activated. Thus, the bulged-nucleotide in the P2–P2a duplex did not provide an essential kink or hinge between P2 and P2a that was required for cleavage activity but, rather, increased the rate of refolding from an inactive to an active ribozyme structure. These data also suggest a model in which P2 and P2a form a coaxial stacked helix of 9 bp, the most likely arrangement being one in which P2–P2a is roughly parallel to P1.

INTRODUCTION

As with protein enzymes, the polymer chain forming a ribozyme must assume a particular three-dimensional structure for it to be active. Ribozymes derived from the RNA of hepatitis delta virus (HDV) (1–3), although relatively small at <100 nt each, can assume either active (native) or one or more inactive but metastable (misfolded) conformations in the presence of divalent metal cations which are required for cleavage activity (4,5). A requirement for unfolding of misfolded structures would account for the slow observed rates and low extents of cleavage measured with various precursor sequences containing HDV ribozymes. Evidence that refolding was required for cleavage included the increased rate and extent of cleavage seen with the addition of urea or formamide, the strong temperature dependence of the rate of cleavage (4) and incremental increases in the extent of cleavage with repeated cycling of reactions between 37 and 100°C (5). In the latter case it is not clear if the cycling was specifically necessary or if rapid cleavage occurred at intermediate temperatures during each cycle. The most straightforward hypothesis to explain the misfolded structures is that they result from non-native secondary (or tertiary) interactions that sequester essential sequences or otherwise distort the active structure. These misfolded structures must be sufficiently stable so as to slow unfolding that is necessary prior to refolding into the active form. It would be predicted that sequence alterations that preferentially stabilize native interactions or destabilize non-native interactions should favor the native structure, while changes that have opposite effects will favor the inactive structure. Such a situation has been described for alternative pairing involving sequences that form P1 and P3 in the Tetrahymena pre-rRNA ribozyme (6,7). However, in the antigenomic HDV ribozyme the sequence that forms the 3′-side of a short duplex, P2a, although non-essential for activity, was found to stabilize both the native and misfolded conformations and the same sequence was involved in the same P2a interaction in both conformations (8).

With the antigenomic RNA of HDV, residual activity can be detected with sequences that terminate 79 nt 3′ of the cleavage site (9–11). However, an additional 10 nt allows the formation of P2 and P2a and the inclusion of these duplexes increases the observed rate of cleavage by at least 103-fold (8,12). P2 and P2a are formed between sequence near the 5′-end (nt 10–19), which would otherwise form part of a bulged loop between P1 and P3, and sequence at the 3′-end of the ribozyme (nt 81–89) (Fig. 1). Within the sequence bounded by the 5′- and 3′-sides of P2–P2a (nt 20–80) reside all the essential nucleotides that form the active site of the ribozyme, exclusive of the 5′-side of P1 and its associated cleavage site. A bulged nucleotide (A14) separates these two short duplexes in the proposed secondary structure and evidence that A14 is not base paired includes its modification by dimethyl sulfate (8) and cutting by RNase U2 (unpublished data).

Although P2a is not required for self-cleavage activity, P2a can further stabilize the correct folding of the RNA (8). When the RNA is preincubated in a moderate concentration of monovalent salt (0.05–0.2 M at 37°C) prior to the addition of Mg2+, P2a favors the folding of the ribozyme sequence into the active RNA–Mg2+ complex. This effect of P2a is not seen if the NaCl and MgCl2 are added simultaneously. In that case, or in the absence of monovalent salt preincubation, the effect of P2a was to decrease the extent and apparent rate of cleavage relative to a
Plasmids and construction of mutants

The ribozyme sequences were from plasmid derivatives of pPEX1, which contains a synthetic antigenomic ribozyme sequence (PEX1) cloned into the phagemid vector pTZ18U (8). PEX1 is nearly identical to the wild-type antigenomic self-cleaving sequence from -3 to +89 (numbering is relative to the cleavage site) except for minor changes that introduced a restriction recognition site in the sequence comprising the non-essential L4. The bulge mutants were generated by oligonucleotide-directed mutagenesis using a uracil-containing single-stranded form of pPEX1 as template (14,15). Plasmid DNA was purified by CsCl/ethidium bromide equilibrium centrifugation.

RNA preparation

Template DNA was prepared by linearizing plasmid DNA with BanI (leaving 94 nt 3′ of the cleavage site). Transcription were as previously described (16,17). Following transcription, EDTA was added to inhibit further cleavage and the RNA was fractionated on polyacrylamide gels. Precursors were eluted from the gel, separated from gel contaminants on a G-25 spin column, ethanol precipitated and stored in 0.1 mM EDTA at -20°C.

Self-cleavage reactions

Radiolabeled precursor RNA (5–50 nM) was heated to 95°C in 0.1 mM EDTA, cooled to 37°C and adjusted to 40 mM Tris–HCl (pH 7.5) and 1 mM EDTA; -3 mM NaCl is present in these reactions. The RNA was then incubated for 10 min at 37°C prior to initiating the cleavage reactions by addition of 0.25 vol of a pre-warmed cocktail of 55 mM MgCl2, 40 mM Tris–HCl (pH 8.0) and 1 mM EDTA; the final concentration of MgCl2 was 11 mM. Where indicated, 0.1 M NaCl or 0.5 mM spermidine was included in the preincubation and in the 5× MgCl2-containing cocktail. Cleavage reactions were at 25°C unless noted otherwise. For the kinetic studies, aliquots were removed at specified times and mixed with a formamide–dye mix containing a 3- to 4-fold molar excess of EDTA to quench the reaction. The precursor and product were separated by gel electrophoresis (6% polyacrylamide gel containing 7 M urea, 0.5 mM EDTA and 0.05 M Tris–borate, pH 8.3). The relative amounts of precursor and 3′ cleavage product were quantified on a phosphorimager (Molecular Dynamics). A correction was made for label in the 5′ product and a first order rate constant, k, was obtained by fitting the data to $F_1 = E(1 - e^{-kt})$ where $F_1$ is the fraction cleaved at time $t$ and $E = F_∞$. The earliest time points taken manually were at 3 or 4 s. Rate constants reported are the average of at least two and usually three independent determinations; variation between independent determinations ranged from 5 to 20%, except where noted. Where the reaction was biphasic, the rate of cleavage for the slow phase of the reaction was calculated both from the slope of the line generated in a plot of the natural log of the fraction uncleaved as a function of time and by fitting the data to the sum of two exponentials using non-linear curve fitting software (KaleidaGraph; Synergy Software).

RNA melts

Thermal denaturation of the RNA was measured using an Aviv UV spectrophotometer. An aliquot of 2.5 ml sample buffer (0.1 M NaCl, 1 mM EDTA, 10 mM PIPES, pH 6.5) was degassed by heating to 95°C in a cuvette with stirring. Precursor RNA (2.5 µl)
was added (~3 μg/ml final concentration) to the heated buffer and the solution was cooled to 25°C. A_{260} was collected at 0.5°C intervals as the RNA was heated from 25 to 95°C at a rate of 0.35°C/min. The sample in the cuvette was stirred constantly. Data collected from three runs with fresh RNA samples each time were combined and normalized. The first derivative was derived after smoothing. Remelting an RNA sample generated curves with the same general shape but with some evidence of possible degradation which we attributed to the high temperatures used. Cooling curves (denatured at 95°C, cooled from 85 to 25°C) indicated that denaturation was reversible under these conditions.

**RESULTS**

**Mutations that remove the bulged A between P2 and P2a**

The ‘wild-type’ antigenomic starting sequence used in these studies is PEX1 (8) which has the potential to form a wild-type 4 bp P2a (Fig. 1). Transcription of BanI-cut pPEX1 plasmid DNA yields a precursor with 8 nt 5′ of the cleavage site and 94 nt 3′ of the cleavage site; the terminal 5 nt at both ends of the precursor RNA are from the vector. Gel-purified PEX1 precursors have been shown to cleave with a first order rate constant of at least 30/min at 37°C in 10 mM MgCl\(_2\) when the RNA was preincubated at 37°C in 0.1 M NaCl prior to the addition of Mg\(^{2+}\) (8). However, if the monovalent salt was omitted during the preincubation step, only 20–25% of the PEX1 RNA cleaved at 37°C, indicating that the P2 pairing could also interfere with cleavage by stabilizing inactive forms of the precursor under some conditions. Precursor RNAs were prepared from plasmids in which A14 was deleted (A14Δ), or a U was inserted between nt positions 85 and 86 (85.1U), or both (A14Δ:85.1U). It was predicted that the additional U could pair with A14 such that mutants A14Δ and 85.1U would generate a continuous P2–P2a helix of 9 and 10 bp, respectively. Consistent with that proposed pairing, protection from cleavage at position 14 was found with end-labeled 85.1U 3′ cleavage product RNA probed with RNase U2 (data not shown). The third variant (A14Δ:85.1U) was expected to reintroduce a discontinuity in the P2–P2a helix and generate the potential for a bulged A in the 3′-side. BanI runoff precursor RNA was prepared from all four plasmids under standard transcription conditions (37°C for 1 h). Relative to PEX1, in which 34% of the precursor cleaved during transcription, there was a consistent but small decrease (~2-fold) in the extent of cleavage for the mutants (17% cleavage for A14Δ, 31% for U85.1 and 20% for A14Δ:U85.1). Alternative transcription conditions were not investigated because sufficient precursor for subsequent studies could be isolated using these conditions.

However, it is noted here that the extent of cleavage during a 60 min transcription was higher than what was seen for the isolated precursors preincubated under the no-added-salt conditions and cleaved under similar conditions as described below.

**Cleavage of purified precursors after no-added-salt preincubation**

The kinetics of self-cleavage were studied using precursor RNA that had been purified by denaturing gel electrophoresis. Cleavage activity was initially tested using no-added-salt preincubation conditions: the RNA was denatured by heating to 95°C in 0.1 mM EDTA and then preincubated in 40 mM Tris–HCl, 1 mM EDTA (pH 7.5) at 37°C for 10 min, followed by 25°C for 5 min before MgCl\(_2\) (11 mM final) was added to start the reaction. For reasons described below, comparison of cleavage activities was generally done at 25 rather than at 37°C to slow the reactions. Under these conditions, the reaction kinetics were biphasic with a rapid phase completed in the first minute of the reaction followed by a much slower phase (Fig. 2A). Such results were consistent with a small fraction of the RNA being in a fast cleaving conformation and most of it in one or more slower cleaving conformations. The extent of cleavage in the initial phase ranged from ~2% for 85.1U to 15% for A14Δ. The rate constants for cleavage in the slow phase of the reactions ranged from 2 × 10^-5/min for 85.1U to 4 × 10^-7/min for PEX1.

**Preincubation in spermidine also activates precursor**

Preincubation of PEX1 precursor in 0.1 M NaCl for 10 min increased the extent of cleavage and resulted in monophase kinetics with a better fit to a single exponential curve (8). In reactions at 37°C the salt-preincubated RNA cleaved with a rate constant in excess of 30/min (data not shown) making kinetic analysis manually unsuitable for detecting small differences in rates. At 25°C, however, both hand pipetting and using a rapid quench instrument gave similar rate constants (8.2 and 10/min, respectively) for the cleavage of PEX1 (Fig. 2B). All subsequent studies were carried out manually at 25°C unless otherwise noted. Preincubation in NaCl also resulted in activation of the three variants (Fig. 2C). The rate constants for cleavage were approximately the same for all of the derivatives; 9.8/min for A14Δ, 7.0/min for 85.1U and 8.9/min for A14Δ:85.1U. While the rate constants showed little variation (~20%) between experiments, there was occasionally day-to-day 2-fold variation in the extent of reaction for PEX1 and some of the mutants. Some variation in extent of cleavage was observed with different preparations of the RNA, but unidentified differences in the preincubation conditions may have also contributed to the variation, so an alternative preincubation procedure was sought.

**Preincubation in spermidine also activates precursor**

Preincubation of PEX1 precursor in spermidine rather than NaCl was found to activate the ribozyme to a similar level but with less day-to-day variation in the extent of cleavage. In previous studies of an antigenomic HDV ribozyme lacking P2a, spermidine did not enhance cleavage and at higher concentrations (≥5 mM) spermidine inhibited cleavage (A.T.Perrotta and M.D.Been, unpublished data). All four ribozymes were activated by preincubation in 0.5 mM spermidine for 5 min at 37°C (Fig. 3A), but of the four, the PEX1 precursor cleaved to the greatest extent. This
Figure 2. Self-cleavage after preincubation in the absence and presence of added NaCl. (A) Radiolabeled precursors were heated to 95°C for 1 min in the absence of NaCl, incubated at 37°C for 10 min and then cooled to 25°C for 5 min before initiating the cleavage reaction by the addition of MgCl₂. (B) PEX1 precursor was treated as before except after cooling to 37°C the conditions were adjusted to include 0.1 M NaCl, then incubation at 37°C was continued for 10 min before cooling to 25°C. (C) Cleavage of the bulge mutants after preincubation in 0.1 M NaCl as described above in (B). Samples at each time point were collected manually.

The bulged A increases the rate of folding of the ribozyme into an activated conformation

The shape of the curves in the cleavage time courses (Fig. 3A–C) suggested that very little refolding occurs once Mg²⁺ is added to start the reaction. Regardless of the preincubation conditions used difference largely disappeared if the preincubation in spermidine was extended to 60 min (Fig. 3B) suggesting that the variants refolded slowly relative to PEX1. Regardless of whether the precursors were preincubated for 5 or 60 min or slow cooled from 95 to 37°C in spermidine (Fig. 3C), they had essentially the same cleavage rates (Table 1). Only the U85.1 precursor consistently cleaved slower than the others, although the difference is relatively small. Assuming that chemistry is at least partially rate limiting under these conditions, the finding that activated precursors for all of the constructs cleaved at essentially the same rate suggested that the bulged A was not required for catalytic activity.

Table 1. Rate constants for self-cleavage in spermidine

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<tr>
<th></th>
<th>5 min</th>
<th>60 min</th>
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<tr>
<td>PEX1</td>
<td>12 ± 2</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
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<tr>
<td>A14Δ</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
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<tr>
<td>85.1U</td>
<td>11 ± 1</td>
<td>8.0 ± 0.8</td>
<td>7.3 ± 0.8</td>
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<tr>
<td>A14Δ:85.1U</td>
<td>12 ± 1</td>
<td>11 ± 1</td>
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All preincubations, including the slow cooling, were in 0.5 mM spermidine as described in the text.

Table 3. Self-cleavage after preincubation in spermidine. (A) Cleavage at 25°C after a 10 min preincubation at 37°C in 0.5 mM spermidine and cooling to 25°C for 5 min before addition of MgCl₂. (B) As (A) except the preincubation in spermidine was increased from 10 to 60 min. (C) Slow cooling (95 to 25°C over ~1 h) in 0.5 mM spermidine before addition of MgCl₂. Rate constants are given in Table 1.
or the particular construct, once Mg$^{2+}$ is added, most of the reaction is completed in <60 s (Figs 2 and 3). This suggests that the Mg$^{2+}$ provides a kinetic lock on the structure and prevents refolding of the misfolded precursor RNA. Thus, the extent of cleavage within the first 60 s of the reaction, after Mg$^{2+}$ is added, is a measure of the amount of active RNA in a reaction and the extent of cleavage in 60 s as a function of preincubation time will reveal the rate of refolding. This approach was used to measure refolding rates and it was found that removing the bulged A or making it part of a base pair slowed the refolding 4- to 5-fold (Fig. 4 and Table 1). Thus, it appears that, in 0.5 mM spermidine, the bulged A facilitates RNA refolding from the inactive to active form.

**Precursor conformers in a native gel**

PEXI precursor was previously shown to be resolved into fast migrating active species and slow migrating inactive species on non-denaturing gels (8). Correlation of the mobility in gels and cleavage activity for each of the bulge mutants provided further evidence for alternative conformations of the precursor that correlate with activity (Fig. 5). In these experiments gel-purified radiolabeled precursors were preincubated for 60 min in no-added NaCl (low Na$^+$), 0.1 M NaCl or 0.5 mM spermidine. MgCl$_2$ was added to half of each sample and incubation was continued for an additional 60 s at 37°C before the samples were loaded onto the gel pre-running at room temperature. The Mg$^{2+}$ was not chelated with the asterisk). The addition of Mg$^{2+}$ resulted in disappearance of the precursor to the fast migrating species (Fig. 5, band marked with the asterisk). The addition of Mg$^{2+}$ resulted in disappearance of the fast migrating precursor and the appearance of the slower 3’ product. Although accurate quantification of the results from the non-denaturing gels suffers from some smearing of RNA in the lanes, it was nevertheless useful to estimate the percent that shifted in the absence of MgCl$_2$ and the percent that cleaved with MgCl$_2$. The data are consistent with the formation of an alternative, presumably more compact structure in NaCl or spermidine that was capable of rapid cleavage when Mg$^{2+}$ was added. In both NaCl and spermidine, 85.1U precursor formed less active conformations, although not necessarily to the same extent. So while it is possible that the equilibrium distribution between the various conformers is altered by the urea, we presume that the enhanced cleavage is due to an increased rate of conversion of inactive to active conformations. To compare the effect of urea on cleavage of the bulge mutants, the extent of cleavage after 1 min at 37°C with increasing concentrations of urea was measured (Fig. 6A). PEX1 and the bulged U variant (A14Δ:85.1U) cleaved to ∼50% in 5–6 M urea, whereas the

**Figure 4. Rates of refolding estimated from the extent of cleavage as a function of incubation time in spermidine.** The RNA was heated to 95°C for 1 min, cooled to 37°C for 5 min, buffer added [40 nM Tris–HCl (pH 7.5), 1 mM EDTA, 0.5 mM spermidine] and incubated for the times indicated before removing an aliquot and mixing with MgCl$_2$. After an additional 1 min at 37°C the reaction was quenched. Rate constants (per min): PEX1, 0.44 ± 0.05; A14Δ, 0.088 ± 0.009; 85.1U, 0.12 ± 0.02; A14Δ:85.1U, 0.20 ± 0.03.

**Figure 5. Separation of precursor conformers by native gel electrophoresis.** (A) RNA samples were heat denatured and diluted into 40 mM Tris–HCl (pH 7.5), 1 mM EDTA, 4% (v/v) glycerol and 0.05% (w/v) xylene cyanol and preincubated at 37°C for 60 min. To half of each sample, MgCl$_2$ was added and after an additional 1 min at 37°C, both samples were loaded directly onto a pre-running gel. (B) and (C) The preincubation conditions were as in (A) but included either 0.1 M NaCl (B) or 0.5 mM spermidine (C). Values given below each lane are the percent of precursor shifted to the faster migrating species (no Mg$^{2+}$) or the percent of the total that cleaved (+ Mg$^{2+}$) in this particular experiment. The faster migrating precursor species is indicated by the asterisk.

**Removal of the bulged nucleotide stabilizes both inactive and active conformations of the precursor**

The addition of chemical denaturants to the reaction also stimulated cleavage activity of PEX1, although the rate of cleavage was slower than when the RNA was preincubated in NaCl (8) or spermidine. Denaturants might destabilize both active and inactive conformations, although not necessarily to the same extent. So while it is possible that the equilibrium distribution between the various conformers is altered by the urea, we presume that the enhanced cleavage is due to an increased rate of conversion of inactive to active conformations. To compare the effect of urea on cleavage of the bulge mutants, the extent of cleavage after 1 min at 37°C with increasing concentrations of urea was measured (Fig. 6A). PEX1 and the bulged U variant (A14Δ:85.1U) cleaved to ∼50% in 5–6 M urea, whereas the
bulgeless variants (A14Δ and 85.1U) required 8 M urea to cleave to a similar level. We interpret this data to mean that removing the bulge stabilized the misfolded conformation.

To get a better estimate of the contribution the bulged A makes to lowering the energy barrier to refolding, the temperature dependence of the rate of cleavage measured in 5 M urea for PEX1 and the two bulgeless mutants was examined (Fig. 6B). Given sufficient time (10–60 min), the bulgeless mutants cleaved to ∼90% and the data fitted a single exponential curve (data not shown). Thus, for the bulgeless precursors, the kinetic data was consistent with most of the RNA being in an inactive conformation. Together, these results suggest that the extent of cleavage after 1 min (Fig. 6A) reflects mainly the proportion of PEX1 precursor in a fast cleaving conformation whereas for the two bulgeless mutants it reflects both the low fraction of active conformation and the slow rate of conversion of inactive to active conformation.

An Arrhenius plot of the temperature dependence of the rate of cleavage of the bulgeless mutants in 5 M urea provides an estimate of the additional energy required to refold the bulgeless mutants (Fig. 6B). Previously, an activation energy of 75 kcal/mol was calculated for cleavage of misfolded PEX1 in the absence of urea (8) and this was interpreted as evidence for a conformational change in the RNA (18) before cleavage can occur. It would appear that in 5 M urea there is similar barrier (84–85 kcal/mol) for activation of the two bulgeless mutants. The temperature dependence of PEX1 cleavage in 5 M urea was also studied, but because the reactions were biphasic there was less confidence in the rate constants that could be obtained from the data; an activation energy of ∼20 kcal/mol was obtained for the slow phase of the reaction (Fig. 6B). (Estimates for this value ranged from 10 to 40 kcal/mol depending on the assumptions used to calculate the rate constants.) Together these data are consistent with the idea that removing the bulged nucleotide slows the refolding process by stabilizing the misfolded form of the ribozyme. Measuring folding rates as a function of temperature in spermidine without urea, as described for Figure 4, proved unacceptable over a similar temperature range (data not shown).

To determine if removing the bulged A also stabilized the presumably native conformation of the ribozyme, the thermal melting behavior of precursors RNAs was followed in 0.1 M NaCl (Fig. 7A and B). There was cooperative and reversible unfolding of the RNA with melting temperatures of ∼70.4°C for PEX1 and at 74.6°C for the +U mutant. These data were consistent with the idea that removing the interruption by introducing an A-U pair between P2 and P2a also stabilized a folded form of the RNA under conditions where it forms a structure closely resembling the native conformation (which requires Mg²⁺).

**DISCUSSION**

Bulged nucleotides can play critical roles in RNA structure. Bulged adenosines are known to provide sites for protein recognition and binding (19) or to introduce a kink into a duplex (20). In pre-mRNA and group II intron splicing, a putative bulged
removing the bulge was critical for this process. More likely, the wild-type structure facilitated folding but was not required as part of the active structure per se. Removing the bulge, either by deletion of A14 or providing it with a pairing partner, reduced the extent of cleavage under some conditions and slowed the conversion of inactive to active precursors. Yet, when the ribozymes were pre-folded prior to adding MgCl₂, removing the bulge did not substantially alter the subsequent cleavage rates. If a kink or hinge was required to allow essential residues to come together, we might have expected a decrease in the rate of self-cleavage due to distortion of key residues in the active site. This conclusion assumes, first, that P2 and P2a will form a single helix when A14 is deleted and sufficient flexibility would not occur in the bulgeless helix and, second, that disruption of the active site would result in either a change in the rate for the rate limiting step or a different rate limiting step. The main effect of removing the bulge appeared to be consistent with stabilization of both inactive and active conformations and slowing the conversion from misfolded to active forms. Replacing the bulged A with a bulged U in the other strand restored refolding at lower urea concentrations, suggesting that neither the adenosine nor the specific structure of the bulge was required for refolding. While it is possible that the bulged A between P2 and P2a could provide flexibility to facilitate a conformational change without the need for unpairing, the large activation energies seen in conversion of the inactive to the active ribozyme (8) would appear to be more consistent with a requirement for extensive unpairing of duplex elements (18,32). Also, removing the bulged nucleotide is expected to stabilize P2–P2a and we find this change increases the melting temperature of the ribozyme and increases the activation energy for refolding; thus, we hypothesize that the conformational rearrangement requires at least that the P2–P2a duplex be transiently disrupted in the refolding process.

Studies with the Tetrahymena intron ribozyme have revealed two broad classes of interactions that lead to barriers or kinetic traps in RNA folding (33). Non-native interactions typified by alternative pairing of sequences involved in formation of P1 or P3 (Alt P1 and Alt P3) (6,7) can inhibit the formation of P1 or P3 and other essential interactions. In the second class, it has been demonstrated that native interactions can also present barriers to correct folding. Treiber et al. (33) found that in the folding of the Tetrahymena ribozyme the native P4–P6 domain decreases the rate of native P3–P7 domain formation. The case with P2–P2a in the antigenomic HDV ribozyme is more similar to the second example, i.e. a native interaction that can inhibit the formation of the active form of the ribozyme. However, the possible requirement for unpairing that is seen with the P2–P2a duplex in the HDV ribozyme for the switch from the inactive to active conformation is probably not required of the P4–P6 domain in the Tetrahymena ribozyme. The results with the HDV ribozyme suggest that a native kinetic trap is not limited to large multidomain RNAs, but can occur in a smaller tRNA-sized RNA as well.

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