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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 10^3-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 Mg^{2+}, P2a favors the folding of the ribozyme sequence into the active RNA–Mg^{2+} complex. This effect of P2a is not seen if the NaCl and $MgCl_2$ 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

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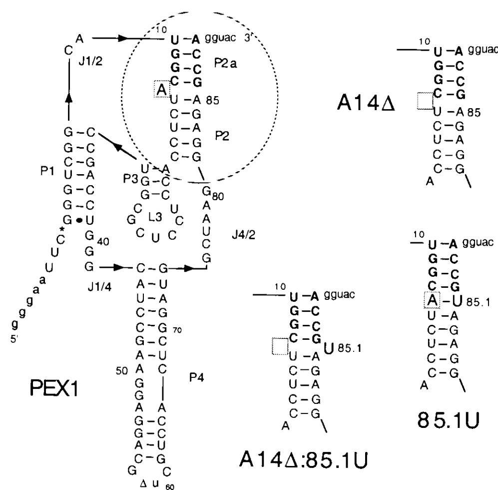

Figure 1. Secondary structures. The sequence of the entire PEX1 precursor is shown in its proposed secondary structure. Only the P2–P2a region (circled) is shown for the three mutants in that the rest of the sequence is the same. Numbering starts 3′ of the cleavage site (*). Lower case letters indicate vector-derived sequences. A non-critical base substitution and deletion in the loop at the end of P4 are indicated by a lower case letter and Δ.

ribozyme lacking P2a. Non-denaturing gel electrophoresis separates two populations of the P2a-containing ribozyme precursor: a faster migrating population that cleaves to completion when Mg^{2+} is added and a slower migrating one that is either inactive or cleaves much slower when Mg^{2+} is added.

In this study we have tested the importance and possible function of the bulged nucleotide, A14, between P2 and P2a. It was found that the bulged A favored folding of the purified precursor into the compact pro-active structure, in the absence of Mg^{2+}, by increasing the rate of refolding from inactive to active conformations; it did not, however, affect the cleavage rate of the active conformations. These data, together with previous results (8), revealed that, although P2–P2a stabilizes the active conformation, the folding process may be slowed or sidetracked as P2–P2a becomes more stable. Thus, the bulged A appears not to participate directly in cleavage or in formation of the active site, nor is it required to provide an essential kink or hinge between P2 and P2a necessary for cleavage. It does appear to reduce a kinetic barrier imposed by P2–P2a for refolding the misfolded ribozyme sequence into active conformations. The data also have implications for the three-dimensional arrangement of P1, P2–P2a and P3 in the antigenomic ribozyme in that they suggest that P2 and P2a form a coaxial stacked helix of 9 bp.

MATERIALS AND METHODS

Enzymes, chemicals and oligonucleotides

T7 RNA polymerase was purified from an overexpressing clone provided by F.W.Studier (13). Restriction endonucleases, nucleotides, ^{32}P-labeled nucleotides, chemical reagents and other enzymes were purchased from commercial suppliers. Oligodeoxynucleotides for mutagenesis were purchased from an in-house synthesis facility.

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 *Ban*I (leaving 94 nt 3′ of the cleavage site). Transcription conditions 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 Na^+ 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 $MgCl_2$, 40 mM Tris–HCl (pH 8.0) and 1 mM EDTA; the final concentration of $MgCl_2$ was 11 mM. Where indicated, 0.1 M NaCl or 0.5 mM spermidine was included in the preincubation and in the 5× $MgCl_2$-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 $F_t = E(1 - e^{-kt})$ where F_t is the fraction cleaved at time t and $E = F_\infty$. 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.

RNA folding

Estimation of the contribution of a bulged A to the stability of a short duplex of the P2–P2a sequence was obtained using MFold v.3.0 by Zuker and Turner on Dr Michael Zuker's server at Washington University (St Louis, MO).

Non-denaturing gel electrophoresis

Non-denaturing gels contained 6% acrylamide:bisacrylamide (29:1) and 50 mM Tris–acetate. Electrophoresis was carried out at room temperature and at sufficiently low power (2 W) to prevent heating.

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 *Ban*I-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 P2a 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 to the P2–P2a helix and generate the potential for a bulged U in the 3′-side. *Ban*I run-off 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^{-4}/min for PEX1.

Cleavage of purified precursors after preincubation in NaCl

Preincubation of PEX1 precursor in 0.1 M NaCl for 10 min increased the extent of cleavage and resulted in monophasic 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

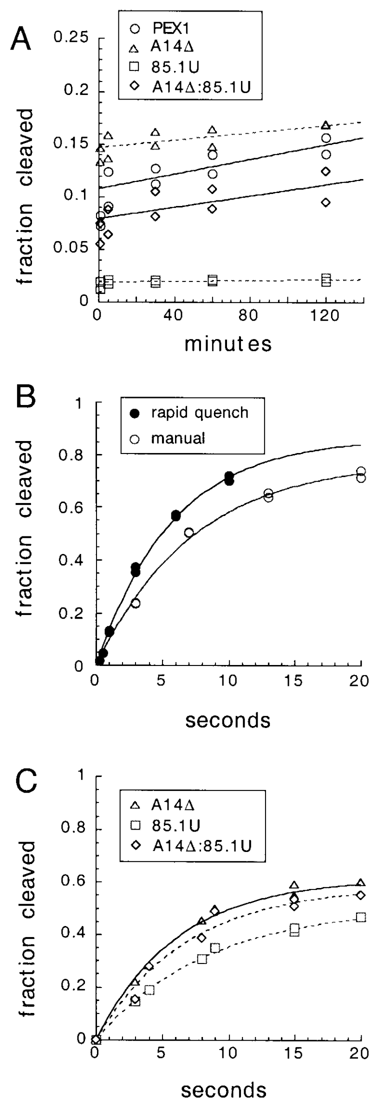

Figure 2. Self-cleavage after preincubation in the absence and presence of added NaCl. (**A**) Radiolabeled precursors were heated to 95°C for 1 min in the absence of NaCl, incubated at 37°C for 10 min and then cooled to 25°C for 5 min before initiating the cleavage reaction by the addition of $MgCl_2$. (**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.

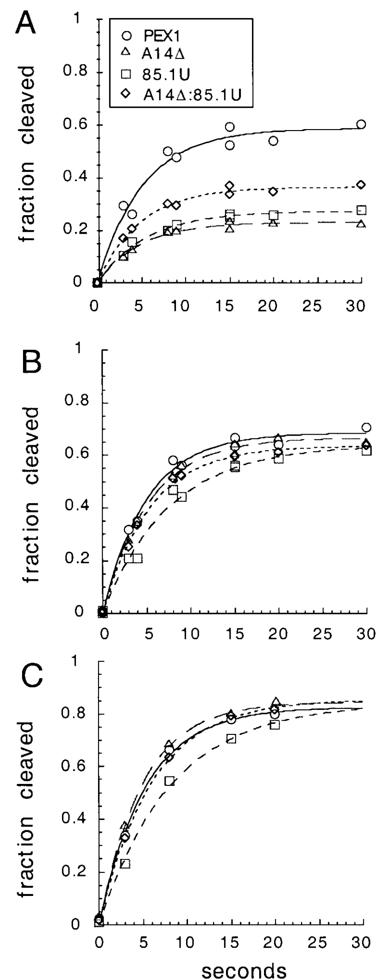

Figure 3. Self-cleavage after preincubation in spermidine. (**A**) Cleavage at 25°C after a 10 min preincubation at 37°C in 0.5 mM spermidine and cooling to 25°C for 5 min before addition of $MgCl_2$. (**B**) As (A) except the preincubation in spermidine was increased from 10 to 60 min. (**C**) Slow cooling (95 to 25°C over ~1 h) in 0.5 mM spermidine before addition of $MgCl_2$. Rate constants are given in Table 1.

Table 1. Rate constants for self-cleavage in spermidine

	Cleavage rates after preincubation in spermidine (per min)[a]		
	5 min	60 min	Slow cool
PEX1	12 ± 2	12 ± 1	11 ± 1
A14Δ	12 ± 1	11 ± 1	12 ± 1
85.1U	11 ± 1	8.0 ± 0.8	7.3 ± 0.8
A14Δ:85.1U	12 ± 1	11 ± 1	10 ± 1

[a]All preincubations, including the slow cooling, were in 0.5 mM spermidine as described in the text.

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.

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 Mg^{2+} is added to start the reaction. Regardless of the preincubation conditions used

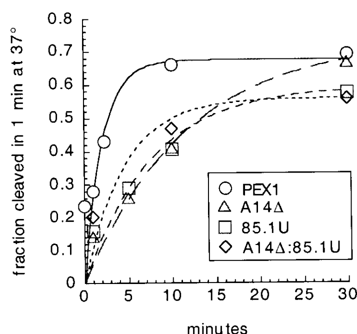

Figure 4. Rates of refolding estimated from the extent of cleavage as a function of incubation time in spermidine. The RNA was heated to 95°C for 1 min, cooled to 37°C for 5 min, buffer added [40 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.5 mM spermidine] and incubated for the times indicated before removing 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.

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 at 37°C, the bulged A facilitates RNA refolding from the inactive to active form.

Precursor conformers in a native gel

PEX1 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 before loading samples onto the gel, but the running buffer contained 1 mM EDTA. Three major species were resolved: the two slower migrating bands were alternative conformers of the precursor, whereas the fastest migrating band in the lanes containing $MgCl_2$-treated samples was 3′ cleavage product. The 5′ cleavage product, because of the small size, was usually not detected in these experiments because it simply ran off the gel.

For all the constructs tested, preincubation in NaCl or spermidine resulted in the conversion of a substantial fraction 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 smaller 3′ product. Although accurate quantification of the results from the non-denaturing gels suffers from some smearing of RNA in

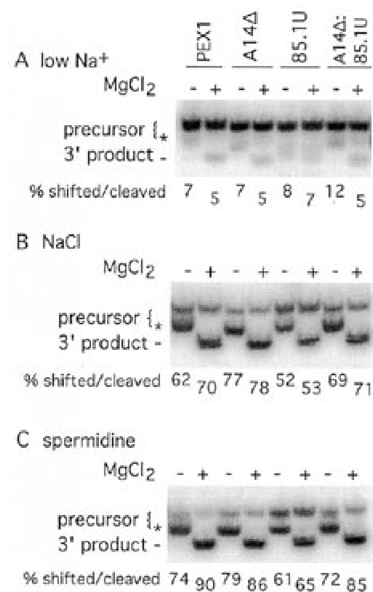

Figure 5. Separation of precursor conformers by native gel electrophoresis. (**A**) RNA samples were heat denatured and diluted into 40 mM Tris–HCl (pH 7.5), 1 mM EDTA, 4% (v/v) glycerol and 0.05% (w/v) xylene cyanol and preincubated at 37°C for 60 min. To half of each sample, $MgCl_2$ was added and 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.

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 of the faster migrating species, but even so, that form of the RNA appeared to cleave to completion. These data are consistent with the hypothesis that the absence of a bulged A in P2–P2a does not interfere with cleavage activity of the native structure but may trap the misfolded RNA and slow the refolding into an active conformation.

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

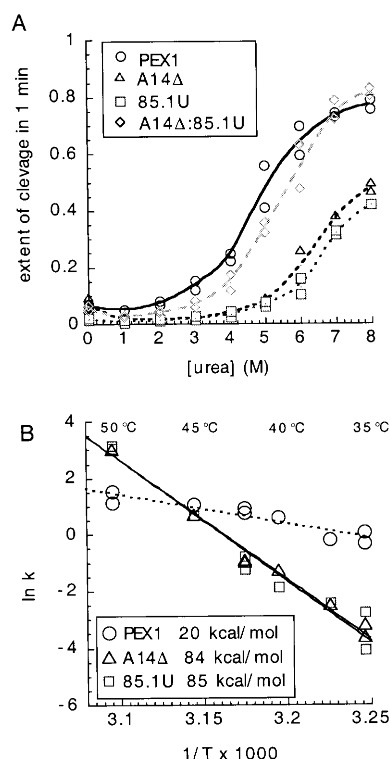

Figure 6. Activation of inactive conformations. (**A**) Urea dependence. The RNA was heat denatured, cooled to 37°C for 5 min and then the conditions adjusted to 40 mM Tris–HCl (pH 7.5) and 1 mM EDTA and increasing amounts of urea. After 10 min at 37°C, $MgCl_2$ was added and incubation continued for 1 min at 37°C before the reaction was stopped with EDTA. The urea concentration during the preincubation was 10% higher than the final concentration shown on the graph. The curves are simply a visual aid. (**B**) Temperature dependence in 5 M urea. The rates of cleavage of precursor RNAs were measured in 5 M urea at temperatures ranging from 35 to 50°C. For the A14Δ and 85.1U ribozymes, the data was fitted to a single exponential; end points ranged from 0.8 to 1. For PEX1, the rate constant for the slow phase was estimated from the slope of ln(fraction uncleaved) versus time after the initial 10 s of the reaction. Activation energies (E_a) were calculated from the slope of ln(k) versus 1/T.

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 and the rate limiting step was slow refolding. PEX1, however, continued to show biphasic behavior, with a fast and slow phase. The proportion of RNA cleaving in the fast phase increased with temperature (41% at 35°C, 58% at 40°C and 74% at 45°C); this result would be consistent with the interpretation that, in 5 M urea, a significant fraction of the PEX1 RNA is in an active 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

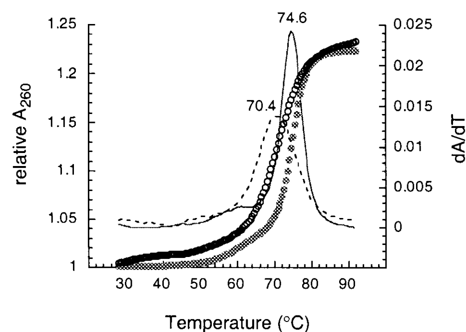

Figure 7. Melting curves for PEX1 and 85.1U precursors in 0.1 M NaCl. Circles are the relative absorbency of the RNA at 260 nm. The curves are the first derivatives and are used to identify the approximate melting temperature. Open symbols and dashed line, PEX1 precursor; closed circles and solid line, 85.1U precursor.

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 Mg^{2+}).

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

A provides the nucleophilic 2′-hydroxyl group for the first step in splicing (21–23). A bulged adenosine is also found adjacent to the guanosine-binding site in group I introns (24). Thus, it is conceivable that the A14 bulge between P2 and P2a in the antigenomic HDV ribozyme is conserved because it contributes to ribozyme activity. It is unlikely, however, that A14 would participate directly as part of the catalytic site because it would not be expected to be close to the cleavage site phosphate (25). The possibility that A14 is important for protein binding or provides another essential function for the virus unrelated to ribozyme activity was not tested in this study. What was tested was the possibility that the A14 bulge provided an essential kink or flexible junction between P2 and P2a. The latter is reasonable given current hypotheses regarding the physical arrangement of P1, P2, P3 and P4 in the antigenomic ribozyme (26–29), i.e. if the single-stranded J1/2 is only 2 nt, it might be expected that a strict collinear arrangement of P3, P2 and P2a would be incompatible with the precise positioning of L3 with portions of J4/2 and the cleavage site phosphate at the end of P1. All three of these regions (cleavage site, L3 and J4/2) are postulated to be in close proximity in the active structure for both the genomic and antigenomic ribozymes (27,29) and a recent crystal structure of the genomic ribozyme 3′ product RNA confirms this arrangement (25). The combination of P2 and P2a is 2 bp longer than P2 in the genomic ribozyme, thus in the antigenomic ribozyme a slight bend or rotation between P2 and P2a could be necessary for the correct orientation of P1 and P3/L3. Before such a function for A14 could be established, it was first necessary to show that this nucleotide affected cleavage of the ribozyme.

The evidence from this study suggested that the bulged A 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 $MgCl_2$, 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 critical for this process. More likely, the wild-type P2–P2a would be expected to be less stable than either of the bulgeless mutants and increasing the stability of the P2–P2a duplex interferes with the folding or refolding process. Thermodynamic data obtained with short duplexes in 1 M NaCl (30,31) predicts that removing the bulged A from P2 and P2a to generate a 9 or 10 bp duplex should provide added stability of approximately –4 or –5 kcal/mol. The melting studies on the ribozyme revealed that removing the bulge increased stability of the ribozyme in 0.1 M NaCl by –8 kcal/mol. We would expect similar contributions to the stability of P2–P2a in both the misfolded and native forms of the ribozyme and, while not quantified, the requirement for higher urea concentrations to get stimulation of activity was consistent with an increase in the stability of the misfolded form upon removal of the bulge. This interpretation is also consistent with the effect of mutations in P2a that destabilized that pairing and resulted in a decrease in the amount of misfolded precursor relative to the wild-type sequence (8).

Several mechanisms could explain how the P2–P2a duplex slows the conversion of the inactive to active ribozyme. The possibility that refolding requires the unpairing and formation of alternative secondary structural elements involving the P2–P2a sequences is inconsistent with the mutagenesis data on P2a (8). Other possibilities include a rearrangement of structural elements other than P2–P2a to generate a different three-dimensional structure. Such a rearrangement may involve alternative secondary structural elements or it is also possible that both the misfolded and the native structures contain essentially the same complement of duplex elements, although in a different three-dimensional arrangement. While unpairing of P2–P2a would not necessarily be required for the rearrangement of those elements, there could be structures where unpairing and repairing of the same duplex greatly facilitates the rearrangement. Such situations might occur if P2–P2a formed or stabilized tertiary interactions in the misfolded structure or if some level of unknotting was necessary. 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 (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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