How RNase R Degrades Structured RNA

ROLE OF THE HELICASE ACTIVITY AND THE S1 DOMAIN

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RNase R, a ubiquitous 3′ to 5′ exoribonuclease, plays an important role in many aspects of RNA metabolism. In contrast to other exoribonucleases, RNase R can efficiently degrade highly structured RNAs, but the mechanism by which this is accomplished has remained elusive. It is known that RNase R contains an unusual, intrinsic RNA helicase activity that facilitates degradation of duplex RNA, but how it stimulates the nuclease activity has also been unclear. Here, we have made use of specifically designed substrates to compare the nuclease and helicase activities of RNase R. We have also identified and mutated several residues in the S1 RNA-binding domain that are important for interacting with duplex RNA and have measured intrinsic tryptophan fluorescence to analyze the conformational changes that occur upon binding of structured RNA. Using these approaches, we have determined the relation of the RNA helicase, ATP binding, and nuclease activities of RNase R. This information has been combined with a structural analysis of RNase R, based on its homology to RNase II, whose structure has been determined, to develop a detailed model that explains how RNase R digests structured RNA and how this differs from its action on single-stranded RNA.

RNase R is a ubiquitous 3′ to 5′ exoribonuclease that, by itself, is able to digest extensively structured RNA molecules (1–4). In vivo, RNase R is primarily a degradative enzyme that acts on mRNAs and on rRNA under certain conditions and that also participates in 3′ maturation of 16S rRNA (1, 5, 6). Other studies have found that RNase R also can complement CsdA, a DEAD box helicase, at low temperatures (7). RNase R is a large (99 kDa), multidomain protein that contains two RNA binding cold shock domains in its N-terminal region, another RNA binding S1 domain in its C-terminal region, and a central nuclease domain. RNase R is subject to a variety of regulatory processes, and its cellular localization and stability are affected by its N- and C-terminal regions (7–11). One of the more interesting properties of RNase R is the presence of an intrinsic RNA helicase activity (11, 12). RNase R and polynucleotide phospho-ylase are the primary nuclease involved in degradation of structured RNAs in vivo (1, 13). Polynucleotide phospho-ylase degrades structured RNA as a part of the RNA degradosome that is associated with an RNA helicase (14–16). In contrast, RNase R appears to act by itself, although the mechanism by which it degrades structured RNA and the role of its intrinsic helicase activity in this process are not yet fully understood.

In earlier work (11), we examined the RNase R helicase activity and found that it is dependent on ATP binding, but not hydrolysis, and that ATP binding occurs only in the presence of a double-stranded RNA substrate. We identified ATP-binding Walker A and Walker B motifs in Escherichia coli RNase R and found that they were conserved in 88% of mesophilic bacterial genera analyzed but were absent from thermophilic bacteria. We also found that although the nuclease activity of RNase R is not needed for its helicase activity, the helicase activity is essential for effective nuclease activity against dsRNA substrates, particularly at lower temperatures and with more stable duplexes. Moreover, the helicase activity utilizes the same catalytic channel as the nuclease activity (11).

Here, we examine in detail the helicase activity of RNase R and its role in the nuclease activity. Using specifically designed substrates, we show that the helicase activity requires a duplex with a 3′ overhang, in agreement with previous findings that the helicase activity utilizes the RNase R nuclease catalytic channel (11) and that substrates with a 5′ overhang bind very weakly in the nuclease channel (2). We also find that RNase R can degrade a duplex substrate with a 3′ overhang, but only in the presence of ATP, demonstrating the importance of the helicase activity for nuclease action, even at 37 °C. Using sequence and model structural analysis, we identified several amino acid residues in the RNase R S1 domain that are important for structured RNA degradation. Mutation of residues Asp716 and Glu717 to alanine renders RNase R unable to bind ATP and to loss of helicase activity. Most importantly, the mutant RNase R lacks nuclease activity against double-stranded RNA but is fully active against single-stranded RNA, demonstrating that these residues are involved specifically in action against the structured RNA substrate. Moreover, conformational analysis using the intrinsic tryptophan fluorescence of RNase R revealed that the conformational change that occurs upon binding of dsRNA and leads to ATP binding does not occur upon mutation of the Asp716 and Glu717 residues. Based on these findings, we present a detailed model that describes the sequence of events that enable RNase R to utilize its intrinsic helicase activity to digest structured RNA.

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Structured RNA Degradation by RNase R

TABLE 1
Site-directed mutagenesis primers

| Mutant primer | Primer sequence |
|---------------|-----------------|
| D716A,E717A   | 5’-GTCGAAAGGCGGTTAAATGCGGCGGCCCAATGACTTTAGGCTG-3’ |
|                | 5’-CAAGCTTAAATGCGGTTAAAGCCCCATATTACGGCTGCG-3’ |
| R718A         | 5’-GCCGTTATATGCGGCGGCCAAACTGACTTTAGGCTG-3’ |
|                | 5’-CAAGCTTAAATGCGGTTAAAGCCCCATATTACGGCTGCG-3’ |

Experimental Procedures

Materials—Mutagenic primers and RNA oligonucleotides were synthesized and purified by Sigma-Genosys. KOD Hot Start DNA Polymerase was obtained from Novagen. DpnI and bacteriophage T4 polynucleotide kinase were purchased from New England Biolabs, Inc. Protein assay dye reagent concentrate for Bradford assays was obtained from Bio-Rad. [γ-32P]ATP was from PerkinElmer Life Sciences. BugBuster protein extraction reagent was purchased from Novagen. SequaGel for denaturing urea-polyacrylamide gels was from National Diagnostics. The Affi-Gel Blue column was obtained from GE Healthcare Life Sciences. All chemicals were reagent grade.

Cloning of RNase R Mutant Constructs—pET44R(D716A,E717A) and pET44R(R718A) were constructed by standard site-directed mutagenesis of pET44R using the corresponding primer pairs listed in Table 1 (2). pET44R(D716A,E717A) and pET44R(R718A) were constructed by site-directed mutagenesis of pET44R using the primers for each mutation listed in Table 1 (11).

Overexpression of RNase R Mutant Proteins—BL21(DE3)pLysS harboring pET44R, pET44R(D716A,E717A), pET44R(R718A), pET44R(D722N,D716A,E717A), or pET44R(D722N,R718A) were grown at 37 °C with shaking to an OD600 of 0.6 in 500 ml of yeast-Tryptone medium supplemented with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, 25 µg/ml kanamycin, and 10 µg/ml tetracycline. Expression was induced by the addition of IPTG to a final concentration of 1 mM. The resulting cell pellet was stored at −80 °C.

Purification of RNase R Mutant Proteins—Full-length wild type RNase R and RNase R mutant proteins were purified from overexpressing cells as described previously (2) with some modifications (11). Although this purification procedure has been shortened from that reported previously (2), based on SDS-PAGE, it leads to wild type and mutant proteins that are each ~90% pure.

Preparation of Oligoribonucleotide Substrates—Oligoribonucleotides were deprotected according to the manufacturer’s instructions. The oligoribonucleotides used were U12C5, A11G10, A12G10, A17G9, A12G9, A14G9, A15G9, A16G9, A17G9, A19G9, A22G9, A11G10, A12G10, A13G10, and A12G12. Oligoribonucleotides were 5’-labeled with [32P] using T4 polyribonucleotide kinase and [γ-32P]ATP. Double-stranded helicase substrates consisting of a 17-base pair duplex with a variable X-nucleotide 3’ overhang (ds17-A<X>) was prepared by mixing 5’-ss17-A<X> with a [32P] complementary ss17 oligoribonucleotide. For nuclease assays the [32P] was on the 5’ end of the longer strand. Strands were mixed in a 1:1.2 molar ratio (unlabeled strand in excess) in the presence of 10 mM Tris-HCl (pH 8.0) and 20 mM KCl, heated in a boiling water bath for 5 min, and then allowed the solution to cool slowly to room temperature.

RNase R Activity Assay—RNase R assays were carried out in 30-µl reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 300 mM KCl, 0.25 mM MgCl2, 5 mM DTT, and 10 µM oligoribonucleotide substrate. The amount of purified enzyme is indicated in the figure legends. Samples were incubated at the indicated temperatures, temperatures, portions were taken at the indicated times or at regular intervals for determination of initial rates, and the reaction was terminated by the addition of 2 volumes of gel loading buffer (95% formamide, 20 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol). Reaction products were resolved on denaturing 7.5 M urea, 20% polyacrylamide gels followed by autoradiography. Quantification was carried out using ImageJ (National Institutes of Health) (17).

Helicase Assay—The RNA substrate used to characterize the helicase activity of RNase R was a 17-base pair duplex with an X-nucleotide 3’ overhang (ds17-A<X>), and was prepared by mixing 5’-32P-labeled U12C5 with the nonradioactive complementary oligoribonucleotide G9A12+X as described earlier. The unwinding activity of RNase R was assessed in 30-µl reaction mixtures containing 50 mM Tris-Cl (pH 7.5), 2.5 mM RNA substrate, 5 mM ATP, 10 mM MgCl2, and 20 mM NaCl at 37 °C (11). The amount of purified enzyme and the incubation time are indicated in the figure legends. The reactions were terminated by addition of a stopping solution to final concentrations of 100 mM EDTA, 10% SDS, and 2 µM of a ssRNA trap consisting of the same strand as the labeled strand of the duplex, which was added to prevent renaneealing of the substrate. Reaction products were analyzed by running a 20% native Tris borate-EDTA polyacrylamide gel followed by autoradiography.

Filter Binding Assay—The double-filter nucleic acid binding assay developed by Wong and Lohman (18) as adapted by Tanaka and Schwer (19) was used. Details of the procedure used were presented previously (11) except that for the current study the [Mg2+] was changed to 10 mM.

RNase R Fluorescence Analysis—For measurement of RNase R intrinsic tryptophan fluorescence, 500-µl reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 20 mM NaCl, 10 µM RNA substrate, and 0.1 µM RNase R D272N or its mutant derivatives were incubated for 30 min at 37 °C. The fluorescence emission spectrum was measured on a PTI fluorometer (Photon Technology International, London, Canada) using an excitation wavelength of 300 nm. Emission was recorded as the mean of three independent measurements from
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In a previous study of the RNase R helicase, a double-stranded RNA substrate (11). However, the mechanism by which the ATP-binding site is generated from the separated Walker A and Walker B motifs and the residues in RNase R that bind the RNA substrate to induce formation of the ATP-binding site were not understood. The studies presented here are designed to more fully understand the helicase activity and to provide mechanistic details about how RNase R digests structured RNA.

Structured RNA Degradation by RNase R

Results

In earlier work, we found that RNase R helicase activity is dependent on ATP binding that occurs only in the presence of a double-stranded RNA substrate (11). However, the mechanism by which the ATP-binding site is generated from the separated Walker A and Walker B motifs and the residues in RNase R that bind the RNA substrate to induce formation of the ATP-binding site were not understood. The studies presented here are designed to more fully understand the helicase activity and to provide mechanistic details about how RNase R digests structured RNA.

**RNase R Helicase Acts Only on Molecules with a 3’ Overhang**—In a previous study of the RNase R helicase, a 17-base pair duplex with a 17-nucleotide 3’ overhang (ds17-A17) was used as substrate (11). Here, we examined whether a double-stranded RNA with a 5’ overhang also is a substrate for the RNase R helicase activity. For this experiment, a 17-base pair duplex with a 5’ overhang, also of A residues, (A17-ds17) was used as the substrate, and it was compared with the same molecule with a 3’ overhang (ds17-A17). As shown in Fig. 1, RNase R exhibited no helicase activity against duplex RNA with a 5’ overhang over a period of 30 min, whereas a substrate with a 3’ overhang was effectively unwound during this period. These data indicate that duplex RNA must have a 3’ overhang to be unwound by the helicase activity consistent with previous findings that the helicase activity utilizes the same catalytic channel as the nuclease activity (11) and that RNA with a 5’ overhang binds weakly to the RNase R nuclease channel (2).

**ATP Binding and Helicase Activity Depend on the Length of the 3’ Overhang**—Inasmuch as the RNase R helicase utilizes the same catalytic channel as the nuclease activity, and because nuclease activity depends on the length of 3’ overhang on the substrate (2), it was of interest to determine whether overhang length also affected ATP binding and consequent helicase activity. Using a filter binding assay (11), we first showed that ATP binding occurs only in the presence of dsRNA, in this case with a 17-nt 3’ overhang (Fig. 2). Based on these data, we estimate that the $K_d$ for ATP is $\sim$1 mM. The amount of [32P]ATP bound was strongly dependent on the length of the 3’ overhang on the duplex RNA substrate (Fig. 3A). At a length of 7-nt or greater, ATP was maximally bound and unaffected by the length of the overhang. However, the amount of ATP bound decreased as the length of the 3’ overhang was shortened below 7 nt. In the presence of an RNA duplex with no overhang or with an overhang of 2 nt, only $\sim$10% of RNase R molecules contained bound ATP.

Likewise, the length of the 3’ overhang affected RNase R helicase activity (Fig. 3B). As with ATP binding, helicase activity was unaffected when the 3’ overhang length was 7 nt or more. In contrast, RNase R helicase activity decreased with decreasing length of the 3’ overhang below 7 nt, and there was no helicase activity with a 0- or 2-nt overhang. These data indicate that helicase activity requires a duplex substrate with at least a 3-nt 3’ overhang and that maximum activity is obtained with a 7-nt overhang.

**Helicase Activity Is Required for Nuclease Activity Against dsRNA Substrates with a Short 3’ Overhang**—In earlier work, we found that RNase R requires a 3’ single-stranded overhang of at least 5 nucleotides to degrade a double-stranded RNA and that a duplex with a less than 10-nt 3’ overhang degrades relatively ineffectively (4). Here, we examined whether the RNase R helicase activity could affect nuclease activity against substrates with short 3’ overhangs. Nuclease activity against RNA duplexes with different 3’ overhangs is shown in Fig. 4. ATP stimulated the nuclease activity, and the stimulation was more pronounced as the length of the 3’ overhang decreased. In fact, although RNase R does not degrade substrates with only 3- or 4-nt long overhangs, these molecules became substrates in the presence of ATP (Fig. 4). These findings indicate that the helicase activity of RNase R expands its nuclease activity to include substrates with even very short 3’ overhangs, most likely by opening the adjacent base pairs to increase the length of the unpaired 3’ sequence.
Conserved Amino Acids in the S1 Domain Are Required for Binding of Duplex RNA—The fact that the ATP-binding site does not pre-exist in the absence of RNA and that only duplex RNA promotes the conformational change that joins the separated Walker A and Walker B motifs to generate a functional ATP binding site (11) strongly suggested the existence of a region specific for interacting with dsRNA. In RNase II, a close homolog of RNase R, three RNA-binding domains (CSD1, CSD2, and S1) come together to form a funnel shape at the top of the nuclease channel that can only accommodate ssRNA (20, 21). In the structural model of RNase R based on the RNase II structure (11), the Walker A motif lies close to and is structurally linked to the S1 RNA-binding domain but is separated by a hinge region. Inasmuch as binding of dsRNA leads to a conformational change that moves the Walker A motif close to Walker B, we postulated that the S1 domain is important for interacting with dsRNA.

Accordingly, we carefully examined the amino acid sequence of the RNase R S1 domain to identify amino acids potentially involved in the interaction with duplex RNA. By sequence analysis, we identified three consecutive amino acid residues near the Walker A motif, Asp$^{716}$, Glu$^{717}$, and Arg$^{718}$ in E. coli RNase R (22), as possible candidates. Analysis of sequences from many diverse organisms using the UniProt database revealed that these three amino acid residues are highly conserved in RNase Rs from different genera of mesophilic bacteria. Using the structural model of RNase R (11), we found that Asp$^{716}$ and Glu$^{717}$ are positioned close to the inside surface of the RNA binding funnel, making them good candidates for interaction with RNA (Fig. 5). Moreover, based on the location of the Walker A motif in the RNase R model structure, it would be possible to change its position to bring it close to Walker B when RNA approaches Asp$^{716}$ and Glu$^{717}$ (Fig. 5). The model structure also suggested that Arg$^{718}$ hydrogen bonds with a conserved aspartic acid residue in the Walker B motif. Based on this analysis, it was likely that Asp$^{716}$, Glu$^{717}$, and Arg$^{718}$ play important roles in RNA binding and in joining the separated Walker A and Walker B motifs to generate a functional ATP binding site.

To test the possibility that these conserved amino acid residues are critical to RNase R function, we generated two mutant proteins, D272N,D716A,E717A and D272N,R718A, and analyzed their catalytic properties (D272N was present to prevent breakdown of RNA during the assays). As shown in Figs. 6 (A and B), the D272N,D716A,E717A mutant protein lacks RNA helicase activity, whereas the D272N,R718A mutant protein displays ~60% of the helicase activity obtained with the RNase R D272N protein, confirming that these residues do, indeed, play a role in RNase R catalysis.

To further examine the role of these amino acid residues, we also measured ATP binding utilizing a filter binding assay to quantify the amount of [$^{32}$P]ATP bound in the presence of saturating amounts of duplex RNA. ATP was bound very poorly to the D272N,D716A,E717A mutant protein, and binding to the D272N,R718A mutant protein was considerably weakened ($K_0$ ~ 5 mM versus 1 mM for WT) compared with RNase R D272N (Fig. 6C). These data support the conclusion that Asp$^{716}$ and Glu$^{717}$ are necessary for generating a functional ATP binding site and that Arg$^{718}$ also contributes to this process.

We next examined how mutation of these conserved residues in the S1 domain affected RNase R nuclease activity. As shown in Fig. 7A, D716A,E717A and R718A mutant proteins were each unaffected in their ability to degrade a single-stranded RNA substrate compared with wild type RNase R. These data show that RNase R retains full nuclease activity even when these conserved amino acid residues in the S1 domain are mutated, indicating that although they do affect ATP binding and helicase activity, nuclease activity against ssRNA is unaltered. In contrast, there was a dramatic effect of the D716A,E717A mutations when the substrate was a dsRNA (ds17−A$_{17}$) (Fig. 7B) or ds17A$_{17}$ (Fig. 7C). The mutant protein displayed greatly reduced nuclease activity compared with wild type RNase R. Essentially the same result was found at an assay temperature of 20 °C instead of 37 °C (Fig. 7D). In each case, ATP did not stimulate the nuclease activity, in contrast to the
stimulation seen with WT proteins, a finding completely consistent with the fact that the mutant protein does not bind ATP (Fig. 6C).

On the other hand, the RNase R R718A mutant protein displays almost the same nuclease activity in the absence of ATP as wild type RNase R against both single-stranded and double-stranded substrates (Fig. 7). However, the ATP stimulation of nuclease activity against double-stranded substrates was considerably less pronounced both at 37 °C and at 20 °C. These findings are consistent with the conclusions that the D716A,E717A mutations affect dsRNA binding, but not that of ssRNA, and that the R718A mutation alters the ability of RNase R to bind ATP and thereby use its helicase activity to stimulate nuclease action.

Double-stranded RNA Induces a Conformational Change That Can Be Measured Using the Intrinsic Tryptophan Fluorescence of RNase R—Our finding that ATP is bound to RNase R only in the presence of duplex RNA (11) implies that the Walker A and Walker B ATP-binding motifs, which normally are separated, must come together, and therefore, that dsRNA, but not ssRNA, induces a conformational change upon binding to RNase R. To analyze this conformational change in more detail, we made use of the intrinsic tryptophan fluorescence of RNase R to directly examine the effect of various RNA substrates and RNase R mutations on this process. Tryptophan fluorescence is an intrinsic property of proteins that requires no protein modification and is particularly well suited to study changes in protein structure that alter the environment surrounding tryptophan residues (23–25).

As shown in Fig. 8A, the intrinsic tryptophan fluorescence of RNase R, which peaks at 378 nm, was totally quenched in the presence of duplex RNA with a 17-nt 3’ overhang. In contrast, there was no change in fluorescence in the presence of ssRNA, even though it also must bind to RNase R because it is an effective substrate (Fig. 7A). Likewise, ATP caused no change in fluorescence as would be expected, because it is unable to bind to RNase R in the absence of dsRNA. dsRNA, by itself, does not fluoresce at these wavelengths. These findings confirm that the quenching of tryptophan fluorescence is a sensitive measure of the conformational change in RNase R structure that occurs upon binding of dsRNA.

Using this methodology, we examined the properties of the S1 domain mutant proteins, D716A,E717A and R718A. As shown in Fig. 8B, although dsRNA completely quenched the fluorescence of WT RNase R (the catalytically inactive D272N derivative is used as WT to prevent destruction of the RNA), no quenching occurred with the D716A,E717A S1 domain double mutant (concentrations of dsRNA as high as 200 μM led to minimal quenching of the mutant protein, whereas 5 μM dsRNA was sufficient to fully quench the WT protein). In fact, fluorescence is somewhat enhanced with the mutant protein, suggesting that mutation of the two amino acid residues leads to greater exposure of tryptophan to the solvent. Nevertheless, these observations support our conclusion that this S1 domain mutant is defective in binding dsRNA, and as a consequence, no conformational change occurs. In contrast, addition of dsRNA to the R718A mutant protein leads to the same quenching of fluorescence as was seen with the WT protein (dsRNA binds...
equally to both proteins). However, although this mutant protein binds dsRNA and undergoes the conformational change, it is unable to bind ATP as well as the WT protein (Fig. 6C), supporting our conclusion that Arg718 is important for binding of ATP but not RNA.

The data presented above showed that the length of the 3’ overhang on dsRNA for lengths less than 7 nt had a profound influence on the ability to degrade such substrates. To determine whether this effect on nuclease activity was also reflected in the ability of the RNAs to induce a conformational change in RNase R, we examined their effectiveness in quenching tryptophan fluorescence. As can be seen in Fig. 8C, 3’ overhangs of 0 or 2 nt did not alter tryptophan fluorescence (increasing the concentration of the 2-nt overhang RNA by 10-fold had no effect), whereas overhangs of 3, 4, or 5 nt had a progressively greater effect on quenching. Once the length of the 3’ overhang reached 7 nt, maximum quenching was observed. These data follow exactly what was observed for ATP binding and helicase
activity (Fig. 3) and for nuclease activity (Fig. 4). These data suggest that RNA molecules with short overhangs bind poorly to RNase R, leading to only partial saturation and fluorescence quenching, and that as the length of the overhang increases, RNA binding is enhanced.

This conclusion was confirmed by the data in Fig. 8D, which shows that for a substrate with a short 4-nt overhang, fluorescence quenching was strongly dependent on substrate concentration, as would be expected for a molecule that binds poorly. Whereas the usual 10 μM concentration used in Fig. 8C led to only partial quenching, complete quenching could be obtained at 75 μM substrate. It should be noted that the presence of 5 mM ATP did not alter the results for the molecule with a 4-nt overhang (data not shown). On the other hand, a substrate with a 17-nt overhang quenched completely at 5 μM (data not shown). These data indicate that the strength of substrate binding depends on the length of ssRNA that can enter the RNA funnel of RNase R and that fluorescence quenching could be used to quantify RNA binding. Most importantly, these data strongly support the conclusion that the conformational change measured by fluorescence quenching is an intimate part of the helicase and nuclease activities of RNase R.

Discussion

The information in this paper provides a more detailed understanding of the relation between the helicase and nuclease activities of RNase R and of the sequence of events necessary for RNase R to digest duplex RNA. Our data indicate that the substrate requirements for inducing ATP binding, for RNA helicase activity, and for nuclease activity are all essentially the same. Each process requires a 3’ overhang and the overhang must be at least 7 nt in length for maximal efficacy. Overhangs as short as 3 nt will work but less efficiently because their binding is too weak to saturate all the enzyme molecules. For molecules with the shortest overhangs that can function as substrates, those 3 or 4 nt in length, they are able to induce the conformational change in RNase R that enables the ATP binding site to form resulting in helicase activity, and this is sufficient to partially open the duplex to lengthen the single-stranded overhang to enable nuclease activity to proceed. In the absence of helicase activity, molecules with only 3- or 4-nt overhangs are not substrates for degradation. Under these conditions the helicase activity is essential for nuclease activity. These findings strongly suggest that the helicase activity depends on interactions of the 3’ overhang with the RNA funnel, whereas nuclease activity requires a longer single-stranded overhang that can reach the catalytic site at the bottom of the channel.

The weak binding of a duplex RNA with a 4-nt overhang can be completely overcome by greatly increasing the RNA concentration, so that all RNase R molecules contain bound RNA. On the other hand, an RNA with 2-nt overhang does not function in any process even when its concentration is greatly increased, setting the minimum length requirement for a 3’ overhang at 3 nt. Most importantly, these data support our conclusion that
FIGURE 7. Effect of S1 domain mutants on RNase R nuclease activity against ssRNA and dsRNA. A, exoribonuclease activity of WT and mutant proteins against single-stranded substrate. Assays were carried out in reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 300 mM KCl, 0.25 mM MgCl₂, 5 mM DTT, 10 μM G₅A₂₉ oligoribonucleotide substrate, and 0.025 μM of enzyme, with and without 5 mM ATP at 37 °C. B, exoribonuclease activity of WT and mutant proteins on ds17-A₁₇ (G₅A₂₉:U₁₂C₅) in the presence or absence of 5 mM ATP. C, exoribonuclease activity of WT and mutant proteins on ds17-A₇ (G₅A₁₉:U₁₂C₅) in the presence or absence of 5 mM ATP. D, exoribonuclease activity of WT and mutant proteins on ds17-A₁₇ (G₅A₁₉:U₁₂C₅) in the presence or absence of 5 mM ATP at 20 °C. For double-stranded substrates, the assays were carried out in reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 300 mM KCl, 0.25 mM MgCl₂, 5 mM DTT, 10 μM oligoribonucleotide substrate, and 0.25 μM enzyme. Portions were taken at the indicated times and analyzed by denaturing PAGE followed by autoradiography. The quantification shown is the average of three experiments ± S.D. and measures disappearance of substrate. △, –ATP; ○, +ATP.
the conformational change, ATP binding, and the helicase activity are all an integral part of the mechanism by which RNase R is able to digest structured RNA.

Our data also indicate that this mechanism differs significantly from that which leads to degradation of single-stranded RNA. With a single-stranded RNA substrate, there is no conformational change and no ATP binding, as would be expected because the helicase activity and strand separation are not needed to digest a single-stranded RNA. Although a single-stranded RNA can directly access the catalytic site at the bottom of the nuclease channel (3, 4), a duplex RNA has to be opened at every catalytic step to enable digestion to proceed through the structured region. Even though the actual nucleolytic step may be identical for single-stranded and double-stranded RNAs, digestion of the latter would require additional steps to separate the strands of the duplex. These considerations would explain why digestion of double-stranded RNA is considerably slower than that of single-stranded molecules (2, 3).

All this new information fits nicely into a structural model of RNase R, based on the crystal structure of the closely related RNase II (20, 21), that expands on our previous proposal to explain how RNase R degrades dsRNA (4, 11). The RNase II structure with a trapped single-stranded RNA revealed that the RNA substrate interactions in the funnel, made up of the two CSD and S1 domains, were mainly to the front part of the S1 domain just above the nuclease channel (Figs. 5 and 9A) (21).
the second strand, whereas the 3′ strand is bound at the front of the S1 domain (Fig. 9B, middle panel). This conformational change, which is detected by the quenching of tryptophan fluorescence, is not triggered by blunt end duplex RNA or by duplexes with only 1- or 2-nt 3′ overhangs, because they bind very poorly to the RNase R funnel; an overhang of at least 3 nt is needed on the 3′ end for tight binding to the S1 domain.

Our data also suggest that the conformational change triggered by the 5′ end of duplex RNA brings together the Walker A and Walker B motifs, and we propose that this structural change is then stabilized by ATP binding (Fig. 9B, middle panel). In RNase R, the Walker A motif is at the C-terminal end of the S1 domain, whereas the Walker B motif is in the CSD2 domain, and so considerable rearrangement of the back of the S1 domain would be required to bring these motifs together. In addition, the residue equivalent to Arg718 in RNase R forms hydrogen bonds with an Asp in the Walker B region in the RNase II structure (residues Arg636 and Asp102 in RNase II). Thus, it is likely that Arg718 is involved in the formation of the ATP binding site. In addition, one of the five Trp residues in RNase R lies very close to this region of the RNA funnel, and consequently, the changes in intrinsic fluorescence that we observe could be directly due to the conformational changes at the Asp716/Glu717 sensor and at the Walker A/B sites.

After the initial conformational change, subsequent steps in the degradation of duplex RNA would require continual opening of base pairs, feeding of the 3′ strand into the nuclease channel, and stabilization of the displaced 5′ strand (Fig. 9B, right panel). We postulate that the conformational change triggered by the 5′ end of the duplex creates a basic channel for the 5′ strand to exit from the back of the RNA funnel and that this channel is stabilized by ATP binding at the Walker A/B motifs.

Interestingly, the RNA binding surfaces of the CSD1/CSD2 domains face the outside of the RNA funnel and away from the single-strand RNA path (21). In RNase R catalysis, these surfaces could be involved in binding the newly displaced 5′ strand of duplex RNA.

This model for RNase R activity (Fig. 9) assumes that the substrate binding funnels in RNase R and RNase II are structurally similar. On the other hand, it is possible that the funnel is somewhat wider in RNase R and can partially accommodate duplex RNA. In fact, this has been suggested in previous models (9). However, because the S1 and CSD domains of RNase R and RNase II are similar at the sequence level, it is unlikely that their funnels differ dramatically in structure. Thus, it is interesting that RNase R is able to digest structured RNA, whereas RNase II cannot. This may be explained because RNase II lacks the 83 residue C-terminal tail in RNase R that extends beyond the S1 domain and contains the Walker A motif (26).

In addition, a more important differentiator between the two enzymes is the much stronger binding of RNA in the catalytic channel that allows RNase R to pull harder on the 3′ strand (4, 20). RNase II may also be unable to open its funnel wide enough to accommodate duplex RNA and may lack features for stabilizing the 5′ strand.

This model for the activity of RNase R fits well with our observation that ATP hydrolysis is not required for helicase activity (11). Instead of ATP hydrolysis, RNase R appears to utilize thermal breathing at the first base pair of the duplex together with tight binding of the 3′ strand on the S1 domain and within the nuclease channel and by holding the 5′ strand at the other end of the funnel to prevent reannealing. The hydrolysis event at the bottom of the nuclease channel “pulls” on the 3′ strand of the RNA to allow the cycle to continue without the

FIGURE 9. Schematic model of the molecular changes in RNase R as it encounters RNA substrates. A, RNA substrates bind the S1 domain (red) and are then fed into a narrow channel in the nuclease domain (green) with high affinity for single-strand RNA. For single-strand substrates, no conformational change in the funnel is needed. B, structured RNA substrates can only partially enter the RNA funnel and require a series of conformational changes. When the duplex region enters the substrate funnel, the 5′ end of the complementary strand encounters the back end of the S1 domain (shown as a red circle, left panel), whereas the 3′ overhang (shown here as 6 nt) begins to enter the catalytic channel. This triggers a conformational change in the funnel (middle panel), and the S1 domain moves to open the funnel to better accommodate the duplex, allowing the 3′ overhang to progress further into the nuclease channel. This conformational change in the S1 domain also enables the Walker A and B motifs (shown as ovals labeled A and B) to come together and bind ATP. We propose that these conformational changes form a channel in the RNA funnel that stabilizes the displaced complementary strand and provides a path for it to spool off as the enzyme processively hydrolyzes the 3′ strand (right panel). RNase R domains are colored as in Fig. 5. N and C represent the N- and C-terminal parts of the protein.
need for any additional input of energy until structured RNA substrate is fully degraded.

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