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The Structure and Enzymatic Properties of a Novel RNase II family enzyme from *Deinococcus radiodurans*

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Abstract

Exoribonucleases are vital in nearly all aspects of RNA metabolism, including RNA maturation, end-turnover, and degradation. RNase II and RNase R are paralogous members of the RNR superfamily of non-specific, 3'→5', processive exoribonucleases. In *Escherichia coli*, RNase II plays a primary role in mRNA decay, and has a preference for unstructured RNA. RNase R, in contrast, is capable of digesting structured RNA and plays a role in the degradation of both mRNA and stable RNA. *Deinococcus radiodurans*, a radiation resistant bacterium, contains two RNR family members. The shorter of these, DrR63, includes a sequence signature typical of RNase R, but we show here that this enzyme is an RNase II-type exonuclease and cannot degrade structured RNA. We also report the crystal structure of this protein, now termed DrII. The DrII structure reveals a truncated RNA binding region in which the N-terminal cold shock domains, typical of most RNR family nucleases, are replaced by an unusual winged helix-turnhelix domain, where the “wing” is contributed by the C-terminal S1 domain. Consistent with its truncated RNA binding region, DrII is able to remove 3' overhangs from RNA molecules closer to duplexes than do other RNase II type enzymes. DrII also displays distinct sensitivity to pyrimidine-rich regions of ssRNA, and is able to process tRNA precursors with adenosine rich 3' extensions *in vitro*. These data indicate that DrII is the RNase II of *D. radiodurans*, and that its structure and catalytic properties are distinct from those of other related enzymes.

Keywords

Exoribonuclease; hydrolytic nuclease; RNA degradation; RNA processing

Introduction

Exoribonucleases play an important role in nearly all aspects of RNA metabolism. The maturation and quality control of stable RNA molecules, and the degradation of all RNA,

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require the action of exoribonucleases. Of the eight exoribonucleases in the well-studied *E. coli*, 3 processive nucleases are primarily responsible for RNA degradation. RNase II (EcII) and polynucleotide phosphorylase (PNPase) extensively degrade single-stranded RNA, though with somewhat different specificities.^{1,2} RNase R, in contrast, degrades structured RNA, including defective tRNA and rRNA, and structured regions of mRNA.³ PNPase, as part of the degradosome complex, is also able to degrade structured RNA.⁴ In *E. coli*, RNase II is the major exoribonuclease, responsible for up to 90% of the hydrolytic activity in a cell lysate.⁵

RNase R and RNase II both are members of the RNR exonuclease superfamily, consisting of large, multidomain proteins, with a shared, linear domain architecture.⁶ RNR family nucleases typically have two N-terminal, RNA binding, cold-shock domains (CSD1; CSD2), an exoribonuclease domain (RNB domain), and a C-terminal S1 domain. The nuclease domain includes four characteristic sequence motifs, with Motif IV originally suggested as an RNase II signature.⁷ Nearly all genomes sequenced to date include at least one RNR family nuclease. Organisms with two RNR family members usually contain one RNase R subclass and one RNase II subclass exonuclease.⁶

Deinococcus radiodurans is a radiation-resistant bacterium containing 2 RNR family members, termed DrR63 (UniProt entry Q9RYD0; 461 residues; gene DR_0020) and DrR77 (UniProt entry Q9RXG0; 760 residues; gene DR_0353) by the Northeast Structural Genomics Consortium. The shorter member, DrR63, contains sequence and structural features reminiscent of RNase R. For example, DrR63 has an arginine (Arg-344) in conserved motif IV that lines the nuclease channel in the RNase R subclass, rather than the lysine seen in RNase II type nucleases. This arginine residue was recently suggested to be critical in the catalytic properties of *E. coli* RNase R.⁸ Interestingly, DrR63 lacks the canonical N-terminal RNA binding CSDs seen in other RNR family members (Fig. 1a).

In this report, we present the crystal structure and biochemical properties of *D. radiodurans* DrR63 and show that it is an RNase II-type enzyme (DrII) that is unable to degrade structured RNA. The overall architecture of DrII is similar to that of EcII, although it has several unique features. Rather than incorporating two CSD domains, as seen in the EcII structure,^{9,10} the DrII N-terminal region folds into a helix-turn-helix (HTH) motif. An extension from the C-terminal S1 domain interacts with the HTH domain in a manner reminiscent of the winged-HTH (wHTH) motif seen in some RNA binding proteins.¹¹ The wHTH forms an open surface that is poised just above the entrance to the narrow channel leading to the DrII active site, and likely forms a putative RNA binding region. The open RNA binding patch of DrII contrasts with the single-strand specific RNA binding clamp seen in EcII.^{9,10} Our biochemical studies show that, as a consequence, DrII is able to approach closer to an RNA duplex than EcII. Furthermore, DrII displays distinct substrate specificity, including sensitivity to pyrimidine-rich regions. The truncated DrII structure coupled with its unusual specificity may help in elucidating the enzyme's function *in vivo*.

Results

Full length DrII was expressed in *E. coli* with a C-terminal, His₆ tag for purification. The 50.6 kDa protein crystallized in both the triclinic (P1) and the hexagonal (P6₃) space groups (Table 1). Structures were solved for the respective space groups to 1.8 Å (PDB ID: 2R7D) and 2.7 Å (PDB ID: 2R7F) using single wavelength anomalous diffraction (SAD). The structures of DrII in the two crystals forms are very similar (backbone RMSD between molecule A in 2R7D and 2R7F is 0.56 Å), and all figures and discussions in this report are based on molecule A from the higher resolution 2R7D PDB entry.

The Structure of *D. radiodurans* RNase II

The *D. radiodurans* RNase II crystal structure reveals 3 distinct domains: an N-terminal HTH domain; a central catalytic or RNB domain; and a C-terminal S1 domain (Fig. 1a). The N- and C-terminal domains come together to form an open RNA binding surface, resting above the central nuclease domain (Figs. 1b and 1c). The RNA binding surface leads to a narrow catalytic channel containing the sequestered active site where a bound, divalent cation is coordinated by several aspartate residues.

The RNA binding Path to a Sequestered Nuclease Channel

RNase II is highly processive on single-stranded RNA,^{12,13} and appears to bind such substrates at two locations: a catalytic site that binds 3' nucleotides and an upstream anchor site.¹⁴ To understand the likely path of RNA on DrII, we took advantage of co-crystal structures of two RNR family exonucleases: *E. coli* RNase II¹⁰ and *S. cerevisiae* Rrp44, the catalytic subunit of the eukaryotic exosome.¹⁵ A superimposition of the 13 nucleotide (nt) RNA fragment from the EcII co-crystal structure¹⁰ on DrII positions the 3' end of the single strand RNA at the catalytic center, and threads the RNA correctly through the narrow catalytic channel (Fig. 1c). This is as expected since the nuclease domains of DrII and EcII are very similar in structure, with Ca RMSD of 1.83 Å (measured over the homologous nuclease and S1 domains). The 5' region of the RNA passes very closely to the positively-charged surface at the interface between the DrII S1 and the HTH domains, and likely defines the RNA binding path for this exonuclease (Fig. 1c). The overall length of the RNA binding path in DrII is thus likely to be similar to that seen in EcII, with the nuclease channel sequestering about 20 Å of the 3' end in a manner identical to EcII. However, the 5' part of this RNA path is quite distinct between the two enzymes, with DrII displaying an open architecture and a truncated N-terminus, while in EcII a narrow clamp-like arrangement is observed.⁹

The EcII clamp measures 8–9 Å across, wide enough only for single-stranded substrate access.⁹ This clamp thus restricts access of duplex RNA from the entire RNA binding path. In the absence of a similar clamp, DrII should be able to accommodate duplex RNA right up to the entrance of the nuclease channel, approximately 6–7 nt closer than in EcII (Fig. 1c). The more accessible catalytic channel of DrII should allow duplexes with shorter 3' overhangs to enter the nuclease channel, similar to the behavior of an N-terminally truncated EcII.⁹ *In vitro* assays described below, using duplex substrates with 3' overhangs, corroborate this structural prediction.

The architecture of the DrII nuclease and S1 domains resembles *E. coli* RNase II (Fig. 1c). However, DrII appears to have only one of the three canonical RNA binding domains (two N-terminal CSDs, and a C-terminal S1 domain) that are present in EcII. Therefore, the closed clamp-like arrangement formed by the three EcII RNA binding domains does not occur in DrII. The absence of a closed ring or clamp-like architecture is somewhat surprising, as most other RNA decay machines such as the eukaryotic/archaeal exosomes, degradosomes and PNPase contain RNA binding rings that funnel substrates to sequestered active sites.^{9,16} Presumably, these closed ring or clamp-like structures were selected to constrain substrate access to the sequestered active site residues.

The Nuclease Channel and Active Site

Though somewhat smaller in sequence length, the catalytic domain of DrII superimposes well with that of EcII. The HTH domain leads to a long extended surface loop region that reaches the bottom of the nuclease domain. The base of the catalytic channel is a 5-stranded, anti-parallel beta sheet containing the putative active site. RNA docking shows that 6 nt of ssRNA can be accommodated within the DrII nuclease channel, and highlights several

putative RNA binding residues (Fig. 2a). His-327 and Met-330 are positioned near the top of the channel and poised to interact with the RNA between nucleotides 4 and 5, while Gln-267 and 337, along with Glu-242, may contact nucleotides 3 and 4. At the base of the channel lies Motif IV, containing the signature RRY sequence. Arg-343 and Arg-344 of this motif form the first 2 steps of a basic ladder (Fig. 2a). While these residues are positioned similarly in DrII and EcII, Arg-344 in DrII is replaced with a Lys in the EcII enzyme. *E. coli* RNase R, however, maintains the RRY signature. An additional tyrosine (Tyr-145), conserved in EcII, lines the exit channel to offer base stacking interactions with the 3' terminal nucleotide. Finally, we see a single divalent cation coordinated by several aspartates, including Asp 93 and Asp 102, at the DrII putative catalytic center which likely also binds a second divalent cation as postulated for EcII.¹⁰ In summary, the overall architecture of the DrII channel and active site is similar to EcII, suggesting a similar hydrolytic mechanism in both enzymes.

The HTH and S1 domains form an open, RNA binding patch

In the EcII structure, two N-terminal cold shock domains (CSDs) come together with a C-terminal S1 domain to form an RNA binding clamp. In DrII, the N-terminal CSDs are entirely absent, resulting in the loss of the clamp-like arrangement seen in EcII. Instead, the DrII N-terminal region folds into a HTH domain not previously seen in X-ray structures of other RNR family enzymes. Interestingly, secondary structure predictions for *E. coli* RNase R (EcR) also suggest an N-terminal HTH domain.¹⁷ The DrII HTH domain is formed at the N-terminus of the protein through the packing of 4 α -helices (H1–H4; Fig. 2b). The function of the DrII HTH domain is unknown, but some duplex RNA binding HTH domains have been described previously.^{11,18–20} The HTH domain seen in DrII is rather unusual since an extended S1 domain loop that connects 2 β -strands (S1 and S2 in Fig. 2b) stretches towards the HTH edge (helix H3). This interaction effectively provides a β wing for the DrII HTH domain, creating an inter-domain winged-HTH.

Similar β -wing loops in wHTH domains have been proposed as recognition sites for the minor groove in duplex RNA,¹¹ and for strand separation of the first DNA base pair by the RecQ DNA helicase.²¹ In ribosomal protein L11, the β -wing loop was seen to be flexible upon RNA binding, which was promoted by a cis-trans proline isomerization at the center of the loop.²² Interestingly, the DrII β -wing loop also contains a central proline (Pro-452). Two arginines (Arg-455 and Arg-457) along with a hydrophobic methionine (Met-454) form a potential RNA binding motif in the β -wing's S1 strand and connecting loop (Fig. 2b).

The positioning of helix H3 at this interface is aided by a 6 residue loop connecting H2 and H3. In RNA binding wHTH domains, helices H1 and H2 often precede the β -wing which is then followed by additional helices.¹¹ For example, in the elongation factor SelB, a surface between H2, H3, and the β -wing may be involved in RNA binding contacts.^{19,20} In DrII, electrostatic analysis and substrate docking (Fig. 1c) support a similar prediction that RNA binding will occur at the interface of the S1 and HTH domains, with a large basic patch corresponding to the β -wing region (Figs. 1c and 2b) interacting with the docked RNA substrate. Residues in helix H3 of the HTH domain are also likely to provide significant RNA binding contacts. Residues Arg-46, Leu-45, and His-41 all reside on the solvent accessible face of H3 that interacts with the β -wing loop (Fig. 2b). It is also possible that H3 recognizes the major groove of distorted A-form RNA, as has been proposed for other RNA binding-wHTH domains.^{11,22}

DrR63 is an RNase II type enzyme

To determine if DrR63 behaves like an RNase R or RNase II-type enzyme, purified DrR63 was assayed using a G-C rich, 17 bp duplex RNA, with a 17 nt 3' poly(A) overhang (ds17-

A₁₇). Purified EcII, which is sensitive to secondary structure, and EcR, which can digest duplex RNA with an appropriate 3' overhang, were also assayed for comparison.²³ Fig. 3a shows that DrII can digest the 17 nt ssRNA overhang of this substrate, but stalls 3–5 nucleotides prior to the duplex. EcII also degrades the overhang, but pauses 7–10 nucleotides upstream of the duplex. RNase R, on the other hand, processively digests the entire substrate. Based on these data, DrII is likely the RNase II-type enzyme of *Deinococcus radiodurans* (DrII) due to its sensitivity to secondary structure. Interestingly, DrII is much less active than EcII (compare the concentrations of enzyme used in Fig. 3a needed to obtain comparable activity).

Action on a short 3' overhang

DrII appears able to approach closer to a duplex than EcII (3–5 nt versus 7–10 nt, respectively). To test the minimum overhang requirements for DrII, the enzyme was assayed using ds17-A₆ as the substrate under conditions identical to those in Fig. 3a. EcII and EcR were again assayed for comparison. Interestingly, DrII trims 3 nt from the overhang, generating ds17-A₃. This trimming process appears as a descending ladder, indicating a distributive, exonucleolytic mechanism of nucleotide removal (Fig. 3b). EcII, can only remove 1 nt from the 6 nt overhang, whereas EcR processively digests the entire substrate (Fig. 3b). Using a substrate with a 5 nt overhang (ds17-A₅), DrII removes primarily a single nucleotide, leaving a 4 nt overhang, but also generates some product with a 3 nt overhang. EcII is inactive on this substrate at the indicated concentrations. Again, EcR, digests the entire substrate (Fig. 3c). These data indicate that DrII prefers a 3' overhang of at least 6 nucleotides, although it has some activity on substrates with only a 5 nt overhang.

Sensitivity to pyrimidines in single-stranded RNA

As shown above, DrII is highly active on duplex RNA with poly(A) overhangs of sufficient length (Figs. 3a and 3b). To further characterize the specificity of DrII, the enzyme was tested on a 34 nt single stranded substrate (ss17-A₁₇) (Fig. 4a). *E. coli* RNase II and R were used for comparison. DrII is highly processive on the poly(A) region of the substrate, but it stalls as it approaches the pyrimidine rich region, accumulating 13–17 mer products (Fig. 4a). DrII also becomes distributive as the substrate shortens. In contrast, EcII processively degrades the ss17-A₁₇ substrate, generating limit products of 4–5 nt, although it does show slight stalling at 9–10 nt, a C rich region. EcR efficiently degrades the entire substrate generating 2–3 nt limit products. Hence, DrII is much less active on ss17-A₁₇ than either EcII or EcR and apparently pauses when a C-U rich region is within a few nucleotides from the catalytic core.

To confirm and extend these results, DrII was assayed on ss17-A₆, a similar single-stranded substrate, but with a shorter poly(A) tail (Fig. 4b). DrII appears to digest only a few nt before stalling at 19–21 mer. After 30–60 minutes, products in the 6–17 nt nucleotide region accumulate, suggesting a highly distributive mechanism. These products are largely composed of the C-U rich region of the substrate. In contrast, *E. coli* RNase II and RNase R are both highly processive, yielding limit products of 3–4 nt, and 2–3 nt, respectively. These results further demonstrate the enhanced sensitivity of DrII to pyrimidine rich regions, as compared to EcII and EcR. This sensitivity appears to occur even when pyrimidines are only a few nucleotides from the 3' end.

Effect of CCA at 3' terminus

E. coli RNase II has the ability to mature the 3' end of pre-tRNAs, albeit rather poorly.²⁴ We decided to test DrII's sensitivity to a CCA overhang, a 3' sequence present in all tRNA and some of its precursors. A 25mer, G₅A₁₂CCA-A₅ was annealed with a complementary C₅U₁₂, yielding a substrate with an 8 nt overhang (CCA-₅). EcII was used for comparison

(Fig. 5). As can be seen, DrII acts as a distributive exonuclease on this substrate, generating a major product with a 3–4 nt overhang. This would correspond to the 3' sequences CCA and CCA-A. EcII poorly digests this substrate, generating a mixture of products with 5–8 nt overhangs (CCA-A₂ to CCA-A₅).

Processing of *D. radiodurans* tRNA precursors

To analyze the role of DrII in 3' tRNA processing, the purified enzyme was incubated with tRNA precursors synthesized *in vitro* by T7 RNA Polymerase. Since all *D. radiodurans* tRNAs except one have CCA encoded, we transcribed tRNA containing this sequence. We have already shown that DrII displays a preference for A-rich regions in single-stranded substrates. Therefore, we compared DrII activity *in vitro* on two tRNAs with dissimilar precursors from the *Deinococcus* genome. The tRNA_{Arg}^{TCT} contains a 7-nt, adenosine rich, 3' overhang. In contrast, the tRNA_{Ala}^{CGC} species has a 7 nt, pyrimidine-rich, 3' overhang. DrII exonucleolytically degrades the adenosine rich, 3' tRNA trailer of tRNA_{Arg}^{TCT} in a distributive fashion by 30 minutes (Fig. 6a). At the conclusion of this reaction, the enzyme pauses 2 nt upstream of the CCA, corresponding to a 6 nt overhang to the acceptor stem. Heterogeneity in the transcription reaction caused the starting material to appear as a doublet (the full length substrate is indicated by an arrow). Interestingly, the +2 residue corresponds to a C residue, leaving an overhang sequence of ACCAAC (see Discussion). DrII was next assayed at identical enzyme and substrate concentrations on tRNA_{Ala}^{CGC} (Fig. 6b). After 120 minutes, none of the substrate had been digested, suggesting that DrII is essentially inactive on this pre-tRNA. These results indicate that DrII can process certain tRNA precursors, but is highly sensitive to pyrimidines in the precursor region.

Generation of a mature 3' termini on an *E. coli* pre-tRNA

We hypothesized that the inability of DrII to remove the final 2 nt 3' to CCA in tRNA_{Arg}^{TCT} (Fig. 6a) was due to the presence of 3 C residues in the catalytic channel at the same time (ACCAAC). Therefore, we generated a tRNA precursor, *E. coli* tRNA^{SelC(p)}, in which the immediate residues 3' to CCA was adenosine rich (GCCAAAAU). As shown in Fig. 6c, DrII can generate a mature CCA end on tRNA^{SelC(p)}. DrII efficiently removes the first 3 nucleotides in 10 minutes, while the final residue is removed by 30 minutes. In contrast, EcII appears to stall at the +2 nt, but generates a mixture of +1 and +2 species by 60 minutes. These data indicate that DrII is capable of generating a mature CCA sequence, but it is highly sensitive to the base composition of the 3' trailer. Note again that much more DrII is required compared to EcII (Fig. 6c).

Discussion

The RNR family of exoribonucleases can be divided into two distinct types of nucleases.⁶ RNase R is capable of digesting through structured RNA, when provided with a sufficient 3' overhang,²³ while RNase II can only digest single-stranded RNA, stalling when it approaches duplexes.¹² Like *E. coli*, the radiation resistant bacterium *Deinococcus radiodurans* contains two RNR family members (DrR63 and DrR77), suggesting that it harbors both RNase R and RNase II. In this study, we report the X-ray structure of *D. radiodurans*' DrR63 and show that it is an RNase II-type enzyme (DrII). DrII incorporates an open RNA binding surface, in place of the closed clamp-like architecture of EcII (Figs. 1 and 2). An N-terminal HTH domain, not present in the EcII structure, interacts with a flexible loop that connects two β -strands from the conserved C-terminal S1 domain, forming an unusual β -wing common in wHTH domains (Fig. 2b). These domains rest atop a highly conserved nuclease domain with a narrow channel that ends at the sequestered active site (Fig. 2a).

The HTH superfamily consists of a broad array of proteins and is commonly involved in duplex DNA recognition. An early example of an RNA binding HTH domain was described in the structure of ribosomal protein L11.²² While there are a few instances of HTH domains interacting with RNA, a variant of the HTH with a β -hairpin extension (the winged HTH or wHTH) is more common in RNA binding proteins.^{11,18} wHTH domains typically interact with RNA along the surface provided by the β -wing and the helices, rather than the interaction between the recognition helix and the major groove as seen in most DNA binding HTH domains.^{11,25} Some recent examples of RNA binding wHTH domains include the eukaryotic La protein, the elongation factor SelB, the *Drosophila* polar granule protein Oskar, and the CvfB virulence factor.^{11,19,20,25,26} Interestingly, the CvfB virulence factor also requires an RNA binding module composed of interacting wHTH and S1 domains.²⁵ Similar wHTH domains are predicted in other RNR family homologues, and likely assist in duplex RNA binding.¹⁷

It is intriguing that different structures of the RNR family exonucleases have revealed different RNA binding paths or mechanisms (Fig. 1c). In EcII, RNA is channeled through a closed clamp-like arrangement,¹⁰ though an alternate path was also proposed to explain the weak tRNA maturation activity of this enzyme.⁹ In Rrp44, the catalytic subunit of the eukaryotic exosome, the RNA binding clamp is occluded, requiring the RNA to take a different path into the nuclease channel.¹⁵ The DrII structure presents another unique arrangement for binding RNA substrates amongst RNR family exonucleases.

To examine the functional consequences of the open RNA binding surface of DrII, the enzyme was assayed *in vitro* on a series of defined oligonucleotides. Like EcII, DrII pauses several nt upstream of a duplex on a ds17-A₁₇ RNA. However, DrII approaches closer to the duplex (3–5 nt) than EcII (7–10 nt). In contrast, RNase R processively digests the entire substrate (Fig. 3).^{8,23} These data confirm that DrII displays an RNase II-like sensitivity to secondary structure. Since *D. radiodurans* contains two RNR members, it is likely that DrR77 will prove to be the RNase R type nuclease. Interestingly, DrII can approach within 3–4 nt of duplex RNA, even with overhangs as short as 6–8 nt, though the enzyme becomes increasingly distributive as overhang length shortens.

D. radiodurans' RNase II also displays a distinct sequence specificity. The enzyme has a preference for A-rich regions and discriminates against pyrimidines. Like EcII, DrII is largely inhibited by a run of C residues, and stalls in C-U rich regions of a variety of ssRNA substrates that EcR and EcII processively digest. Interestingly, a mutant *E. coli* RNase II lacking its cold shock domains also exhibits greater binding affinity for poly(A) substrates than the full length protein.²⁷ This truncated EcII resembles the DrII architecture, but lacks the HTH domain. This comparison is consistent with DrII's strong preference for A rich sequences.

The *D. radiodurans*' genome lacks several exoribonucleases important for 3' tRNA processing in other organisms. These observations suggest a possible role for DrII in 3' end tRNA maturation. We examined the action of DrII *in vitro* on tRNA precursors containing the CCA motif and a variety of 3' trailer sequences, which suggested that the ability of DrII to mature tRNAs was very sensitive to the precursor sequence. While DrII is able to generate mature tRNA, it strongly prefers A-rich precursors and tends to stall before reaching the mature CCA end when pyrimidines are present.

What could be the *in-vivo* consequences of DrII's truncated architecture? The enzyme has several significant structural and mechanistic properties: the generation of 3–5 nt overhangs 3' to a duplex, a distinct sensitivity to pyrimidines, and the appearance of a unique HTH domain potentially involved in duplex RNA recognition. Previously, the nuclease was

shown to be important in the maturation of 23S rRNA at elevated temperatures.²⁸ Furthermore, the enzyme may play a supporting role in pre-tRNA processing. However, the major role of the enzyme may lie in other processes. RNase II-type enzymes are usually key participants in the turnover of polyadenylated cellular mRNA. In *E. coli*, RNase II has been shown to be protective of certain mRNAs by trimming the 3' poly(A) tail close to stable stem loops, thereby removing the 3' overhang required by 3'→5' exonucleases such as RNase R and PNPase.^{12,29} Since DrII can approach several nt closer to a duplex than EcII, it may be relatively more effective at stabilizing stem loop containing RNAs. This protective RNase II type activity may be an important additional role of DrII and should be investigated further.

Materials & Methods

Cloning, Overexpression and Protein Purification of DrII

Deinococcus radiodurans strain R1 gene DR_0020 (DrR63) was cloned into the pET21d expression vector (Novagen, Inc.) using standard cloning protocols developed by the Northeast Structural Genomics Consortium.^{30,31} pET21d_DrII was transformed into BL21 pLysS (RNase II- R-). A starter culture was inoculated into 1 L of LB supplemented with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, 25 µg/ml kanamycin, and 10 µg/ml tetracycline and grown at 37°C until an A₆₀₀ of 0.5. DrII was induced with 1mM IPTG for 2.5 hours at 37°C. Lysis of resuspended, induced pellets occurred in the presence of protease inhibitors and DNase I. The protein was purified in a simple two-step procedure by application to an IMAC (5 ml HisTrap HP), followed by size exclusion chromatography (S200, GE Healthcare, Inc.). RNase II and RNase R were purified as described previously.^{9,13}

Crystallization and Data Collection

Crystals of SeMet substituted DR_0020 from *D. radiodurans* (DrR63) were obtained in drops containing a 1:1 mixture of protein solution (7.5 mg/mL) and well precipitant solution. The protein crystallized in two different space groups, triclinic and hexagonal (Triclinic condition: 0.5 mM MgCl₂, Tris-HCl, 30% PEG 8000, pH 8.0; Hexagonal condition: 1M Triammonium Citrate, 0.4 mM Bis-Tris Propane, pH 7.0). Both crystals were grown at 18°C by hanging drops vapor diffusion method, cryo-protected with 20% glycerol and flash-cooled in liquid nitrogen. Diffraction data sets were collected on single crystals using the beam line X4C with a MAR scanner 345mm image plate to 1.8 Å (triclinic form), and beam line X4A with a Quantum 4R detector to 2.7 Å (hexagonal form) at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. Data were integrated and scaled with HKL2000 package.³² Matthew's coefficient calculations indicated one monomer per asymmetric unit in hexagonal space group and three molecules in triclinic space group.

Structure Determination and Refinement

The structure of DrR63 was solved by the SAD method using Shelx.³³ The locations of 9 selenium sites per molecule were identified from a 2.8 Å dataset. An experimental electron density map was obtained using ShelxD. After phase refinement, an initial model constructed with resolve³⁴ was extended by ARP/wARP³⁵ and refined with CNS.³⁶ Model building was performed using Coot.³⁷ Several cycles of simulated annealing and minimization were carried out using the CNS program package.³⁶ The R-free was calculated based on 10% of randomly selected data excluded from the refinement. A final R factor of 0.22 and 0.20 and an R-free of 0.24 and 0.24 for triclinic and hexagonal forms respectively were reached. Structure validation was performed with PROCHECK.³⁸ The crystallographic

statistics for data collection and refinement are summarized in Table 1. Protein coordinates have been deposited in the Protein Data Bank (PDB codes 2R7D and 2R7F).

RNA Docking and Electrostatic Potential Maps

RNA docking experiments and electrostatic potential maps generated in PyMol³⁹ using the applicable PDB coordinates. Superimpositions of EcII and DrII were first generated by a rotation search in the DALI server.⁴⁰

Preparation of Substrates

Oligoribonucleotides were deprotected according to the manufacturer's instructions. Sequences of single-strand substrates were analyzed to ensure that no secondary structure was likely to form. The single-stranded oligoribonucleotide substrates were 5'-labeled with ³²P using T4 polynucleotide kinase and [γ -³²P] ATP. A substrate consisting of a 17-base pair duplex with a 17-nucleotide 3' overhang (ds17-A₁₇) was prepared by mixing 5'-³²P-labeled ss17-A₁₇ with the non-radioactive complementary oligoribonucleotide (5'-AAGUGAUGGUGGUGGGG-3') in a 1:1.2 molar ratio in the presence of 10 mM Tris-HCl (pH 8.0) and 20 mM KCl, heating the mixture in a boiling water bath for 5 min, and then allowing the solution to cool slowly to room temperature. All other duplex containing substrates were prepared in a similar fashion.

Electrophoretic Activity Assays

DrII assays were performed in 30 μ l reaction mixtures containing 50 mM Tris-Cl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 5 mM DTT. Enzyme and substrate amounts are indicated on the figures or figure legends. Reaction mixtures were incubated at 37°C, with 4 μ l aliquots removed at indicated time intervals and terminated with 2 volumes gel loading buffer (95% formamide, 20 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Reaction products were run on either a 12% or 20% denaturing PAGE containing 7.5 M urea. For RNase R, assays were performed as described.⁴¹

Synthesis of tRNA Precursors

D. radiodurans or *E. coli* genomic DNA was as a PCR template in using a KOD Hot start DNA polymerase. Forward primers contained a T7 promoter sequence followed by sequences complementary to the applicable tRNA gene. PCR products were used as templates for *in vitro* transcription reactions using the MEGAscript system (Ambion, Inc).

- > The *D. radiodurans* DR_0020 gene product is an RNase II type exoribonuclease (DrII).
- > Crystal structure of DrII reveals a novel wHTH motif likely involved in RNA binding.
- > DrII can remove RNA 3' overhangs closer to duplexes than other RNase II type enzymes.
- > DrII displays distinct sensitivity to pyrimidine-rich regions of ssRNA.

Abbreviations used

CSD Cold Shock Domain

DrII	<i>Deinococcus radiodurans</i> RNase II
EcII	<i>Escherichia coli</i> RNase II
EcR	<i>Escherichia coli</i> RNase R
HTH	Helix-Turn-Helix
nt	nucleotide
PDB	Protein Data Bank
RMSD	root mean squared deviation
SAD	single wavelength anomalous dispersion
wHTH	winged Helix-Turn-Helix

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References

1. Li, Z.; Deutscher, M. Exoribonucleases and Endoribonucleases. In: Curtiss, R., II, editor. *EcoSal Escherichia coli and Salmonella: Cellular and Molecular Biology*. Washington DC: ASM Press; 2004.
2. Régnier P, Arraiano CM. Degradation of mRNA in bacteria: emergence of ubiquitous features. *BioEssays*. 2000; 22:235–244. [PubMed: 10684583]
3. Cheng Z-F, Deutscher MP. An important role for RNase R in mRNA decay. *Mol. Cell*. 2005; 17:313–318. [PubMed: 15664199]
4. Carpousis AJ. The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. *Annu. Rev. Microbiol.* 2007; 61:71–87. [PubMed: 17447862]
5. Deutscher MP, Reuven NB. Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in *Escherichia coli* and *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA*. 1991; 88:3277–3280. [PubMed: 1707536]
6. Zuo Y, Deutscher MP. Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucl. Acids Res.* 2001; 29:1017–1026. [PubMed: 11222749]
7. Mian IS. Comparative sequence analysis of ribonucleases HII III, II PH and D. *Nucl. Acids Res.* 1997; 25:3187–3195. [PubMed: 9241229]
8. Vincent HA, Deutscher MP. Insights into how RNase R degrades structured RNA: analysis of the nuclease domain. *J. Mol. Biol.* 2009a; 387:570–583. [PubMed: 19361424]
9. Zuo Y, Vincent HA, Zhang J, Wang Y, Deutscher MP, Malhotra A. Structural basis for processivity and single-strand specificity of RNase II. *Mol. Cell*. 2006; 24:149–156. [PubMed: 16996291]
10. Frazão C, McVey CE, Amblar M, Barbas A, Vonnrhein C, Arraiano CM, et al. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature*. 2006; 443:110–114. [PubMed: 16957732]
11. Anantharaman V, Zhang D, Aravind L. OST-HTH: a novel predicted RNA-binding domain. *Biology Direct*. 2010; 5:13. [PubMed: 20302647]
12. Coburn GA, Mackie GA. Overexpression, purification, and properties of *Escherichia coli* ribonuclease II. *J. Biol. Chem.* 1996; 271:1048–1053. [PubMed: 8557629]

13. Cheng Z-F, Deutscher MP. Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. J. Biol. Chem. 2002; 277:21624–21629. [PubMed: 11948193]
14. Cannistraro VJ, Kennell D. The processive reaction mechanism of ribonuclease II. J. Mol. Biol. 1994; 243:930–943. [PubMed: 7966309]
15. Lorentzen E, Basquin J, Tomecki R, Dziembowski A, Conti E. Structure of the active subunit of the yeast exosome core, Rrp44: diverse modes of substrate recruitment in the RNase II nuclease family. Mol. Cell. 2008; 29:717–728. [PubMed: 18374646]
16. Lorentzen E, Conti E. Structural basis of 3' end RNA recognition and exoribonucleolytic cleavage by an exosome RNase PH core. Mol. Cell. 2005; 20:473–481. [PubMed: 16285928]
17. Domingues S, Matos RG, Reis FP, Fialho AM, Barbas A, Arraiano CM. Biochemical characterization of the RNase II family of exoribonucleases from the human pathogens *Salmonella typhimurium* and *Streptococcus pneumoniae*. Biochemistry. 2009; 48:11848–11857. [PubMed: 19863111]
18. Draper DE. Themes in RNA-protein recognition. J. Mol. Biol. 1999; 293:255–270. [PubMed: 10550207]
19. Selmer M, Su X-D. Crystal structure of an mRNA-binding fragment of *Moorella thermoacetica* elongation factor SelB. EMBO J. 2002; 21:4145–4153. [PubMed: 12145214]
20. Yoshizawa S, Rasubala L, Ose T, Kohda D, Fourmy D, Maenaka K. Structural basis for mRNA recognition by elongation factor SelB. Nature Struct. Mol. Biol. 2005; 12:198–203. [PubMed: 15665870]
21. Kitano K, Kim S-Y, Hakoshima T. Structural basis for DNA strand separation by the unconventional winged-helix domain of RecQ helicase WRN. Structure. 2010; 18:177–187. [PubMed: 20159463]
22. Xing Y, Guha Thakurta D, Draper DE. The RNA binding domain of ribosomal protein L11 is structurally similar to homeodomains. Nature Struct. Biol. 1997; 4:24–27. [PubMed: 8989317]
23. Vincent HA, Deutscher MP. Substrate recognition and catalysis by the exoribonuclease RNase R. J. Biol. Chem. 2006; 281:29769–29775. [PubMed: 16893880]
24. Li Z, Deutscher MP. Maturation pathways for *E. coli* tRNA precursors: a random multienzyme process *in vivo*. Cell. 1996; 86:503–512. [PubMed: 8756732]
25. Matsumoto Y, Xu Q, Miyazaki S, Kaito C, Farr CL, Axelrod HL, et al. Structure of a virulence regulatory factor CvfB reveals a novel winged helix RNA binding module. Structure. 2010; 18:537–547. [PubMed: 20399190]
26. Dong G, Chakshusmathi G, Wolin SL, Reinisch KM. Structure of the La motif: a winged helix domain mediates RNA binding via a conserved aromatic patch. EMBO J. 2004; 23:1000–1007. [PubMed: 14976553]
27. Amblar M, Barbas A, Fialho AM, Arraiano CM. Characterization of the functional domains of *Escherichia coli* RNase II. J. Mol. Biol. 2006; 360:921–933. [PubMed: 16806266]
28. Chen X, Wurtmann EJ, Van Batavia J, Zybaylov B, Washburn MP, Wolin SL. An ortholog of the Ro autoantigen functions in 23S rRNA maturation in *D. radiodurans*. Genes Dev. 2007; 21:1328–1339. [PubMed: 17510283]
29. Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM, Régnier P. RNase II removes the oligo(A) tails that destabilize the rpsO mRNA of *Escherichia coli*. RNA. 2000; 6:1185–1193. [PubMed: 10943897]
30. Acton TB, Gunsalus KC, Xiao R, Ma L-C, Aramini J, Baran MC, et al. Robotic cloning and Protein Production Platform of the Northeast Structural Genomics Consortium. Methods Enzymol. 2005; 394:210–243. [PubMed: 15808222]
31. Xiao R, Anderson S, Aramini J, Belote R, Buchwald WA, Ciccocanti C, et al. The high-throughput protein sample production platform of the Northeast Structural Genomics Consortium. J. Struct. Biol. 2010; 172:21–33. [PubMed: 20688167]
32. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 1997; 276A:307–332.
33. Sheldrick GM. A short history of SHELX. Acta Crystallogr. A. 2008; 64:112–122. [PubMed: 18156677]

34. Terwilliger TC. Automated main-chain model building by template matching and iterative fragment extension. *Acta Crystallogr D Biol. Crystallogr.* 2003; 59:38–44.
35. Perrakis A, Morris R, Lamzin VS. Automated protein model building combined with iterative structure refinement. *Nature Struct. Biol.* 1999; 6:458–463. [PubMed: 10331874]
36. Brünger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, et al. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* 1998; 54:905–921. [PubMed: 9757107]
37. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol. Crystallogr.* 2004; 60:2126–2132.
38. Lovell SC, Davis IW, Arendall WB, de Bakker PIW, Word JM, Prisant MG, et al. Structure validation by C α geometry: phi,psi and C β deviation. *Proteins.* 2003; 50:437–450. [PubMed: 12557186]
39. DeLano, WL. The PyMOL Molecular Graphics System, Version 1.4. Schrödinger, LLC; 2002. (<http://www.pymol.org>)
40. Holm L, Rosenström P. Dali server: conservation mapping in 3D. *Nucl. Acids Res.* 2010; 38:W545–W549. [PubMed: 20457744]
41. Vincent HA, Deutscher MP. The roles of individual domains of RNase R in substrate binding and exoribonuclease activity. The nuclease domain is sufficient for digestion of structured RNA. *J. Biol. Chem.* 2009b; 284:486–494. [PubMed: 19004832]

(a)

(b)

DrII

Figure 1. Crystal Structure of *D. radiodurans*' RNase II

(a) Domain structure of *E. coli* and *D. radiodurans* RNR family members, as well as yeast Rrp44, color coded as follows: CSD domains, dark or light blue; HTH domain, yellow; catalytic domain, orange; C-terminal S1 domain, red. (b) Crystal structure of *D. radiodurans* RNase II shown in two orientations. The domains are colored as in (a). The green sphere shows the bound metal ion at the DrII putative active site. (c) Comparison of the RNA path (grey) seen in *E. coli* RNase II¹⁰ (top panel) and *S. cerevisiae* Rrp44¹⁵ (bottom panel), with a model of the RNA binding by DrII (middle panel). The RNA path shown for DrII was obtained by superimposing the EcII RNA fragment¹⁰ on the DrII structure. All three molecules are shown in the same orientation, superposed using the catalytic domain. Images

on the left show the backbone secondary structure cartoon colored as in (a), while the right panels show the molecular surface of the enzymes colored by electrostatic charge.

(a)

(b)

Figure 2. Molecular details of the DrII catalytic center and the non-canonical wHTH domain that likely forms the DrII RNA binding patch

(a) Close up view of the putative DrII catalytic center and nuclease channel as a secondary structure cartoon, with residues critical for catalysis and RNA binding shown as sticks. The 3' end of the docked RNA is shown in grey. (b) The wHTH domain region that forms the putative DrII RNA binding patch. The N-terminal helix-turn-helix domain (yellow) forms an interface with an extended S1 domain loop domain (red) to create an open RNA binding surface leading into the DrII nuclease channel. HTH domain helices are labeled H1–H4 beginning from the N-terminus. S1 domain β -strands that form the β -wing structure are labeled S1 and S2. Side chains that line the putative RNA binding surface are depicted as sticks.

(a)

5' - *CCCCACCACCAUCACUUAAAAAAAAAAAAAAAAA-3' (10 μ M)
 3' - GGGGUGGUGGUAGUGAA-5'

Figure 3. DrR63 is an RNase II type enzyme

30 μ l reactions were carried out with the indicated substrate and purified enzyme. 4 μ l aliquots were removed at the specified time points and analyzed by 20% denaturing PAGE. Substrates are shown with an asterisk denoting the 5' 32 P label. **(a)** 10 μ M of a duplex with a 17 nt overhang (ds17-A₁₇). **(b)** 10 μ M of a duplex with a 6 nt overhang (ds17-A₆). **(c)** 10 μ M of a duplex with a 5 nt overhang (ds17-A₅). Minor bands below 20-mer in panels b and c are contaminating degradation fragments, also present at time 0.

Figure 4. DrII sensitivity to a stretch of pyrimidines

(a) Assays were carried out as described under “Materials & Methods” with 10 μ M ss17-A₁₇ substrate and the indicated enzyme concentrations. Aliquots were taken at the indicated times and analyzed by denaturing PAGE. **(b)** DrII activity on ss17-A₆. The use of high percentage PAGE gels allows us to get single nucleotide resolution, and count up from the limit products to arrive at the nt sizes shown on the right. The size of limit products of EcII and EcR are well established.^{23,41}

Figure 5. Effect of CCA at a 3' overhang on DrII activity

An RNA substrate containing a CCA sequence, followed by five A residues, at the 3' terminus of [32 P] G₅A₁₂ was annealed to the complementary C₅U₁₂ as described in "Materials & Methods". The 17 and 18 mer bands at time 0 correspond to existing substrate breakdown products present prior to the addition of enzyme.

Figure 6. The 3' processing of tRNA precursors

Purified DrII was incubated with *in vitro* transcribed **(a)** tRNA^{Arg} and **(b)** tRNA^{Ala} from *D. radiodurans* uniformly labeled using $\alpha^{32}\text{P}$ -ATP. The 7 nt precursor sequence is indicated 3' to the encoded CCA. Portions were removed at indicated time points and run on a 6% denaturing PAGE sequencing gel. The lanes labeled CCA correspond to *in vitro* transcribed mature tRNA (also marked as M). **(c)** Comparison of DrII and EcII activity on an *in vitro* transcribed tRNA^{Ser} precursor from *E. coli*. DrII and EcII were incubated with 5 μM tRNA^{Ser} with portions removed at the indicated time points and run on a 6% denaturing PAGE sequencing gel.

Table 1
Summary of crystals parameters, data collection and refinement

Values in parentheses are for the highest resolution bin.

Space group	P1	P63
Molecules per asymmetric unit	3	1
V_M (Å ³ Da ⁻¹)	2.88	2.78
Unit Cell	a = 65.762 Å, b = 92.205 Å, c = 92.345 Å $\alpha = 60.08^\circ$, $\beta = 89.57^\circ$, $\gamma = 71.11^\circ$	a = 92.143 Å, b = 92.143 Å, c = 118.562 Å $\alpha = \beta = 90.0^\circ$, $\gamma = 120.0^\circ$
Wavelength (Å)	0.979	0.979
Resolution (Å)	50–1.8 (1.8–1.86)	50–2.7 (2.7–2.87)
Temperature (K)	100	100
Unique reflections	308057	34287
Mean I/ σ (I)	14.0	13.3
Sigma Cutoff	0	0
Completeness (%)	96.0 (88.0)	98.7 (96.7)
Redundancy	2.0 (1.7)	8 (7.1)
$R_{\text{merge}}^{\#}$	0.044 (0.307)	0.088 (0.068)
R_{cryst}^+	0.221	0.201
R_{free}^*	0.243	0.244
RMSD Bond lengths (Å) Bond angles (°)	0.009 1.54	0.007 1.30
No. of residues No. of ions	1379 3	459

$$^{\#} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

$$^+ R_{\text{cryst}} = \frac{\sum_{hkl} \|F_{\text{obs}} - |F_{\text{calc}}|\|}{\sum_{hkl} |F_{\text{obs}}|}.$$

* R_{free} is calculated in same manner as R_{cryst} except that it uses 10% of the reflection data omitted from refinement.