Biochimica et Biophysica Acta 1820 (2012) 1543–1552

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

The only exoribonuclease present in *Haloferax volcanii* has an unique response to temperature changes

Rute G. Matos [a], Eduardo López-Viñas [b,c], Paulino Goméz-Puertas [b], Cecília M. Arraiano [a,*]

[a] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av da República, 2780-157 Oeiras, Portugal.
[b] Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), C/ Nicolás Cabrera, 1, Cantoblanco, 28049 Madrid, Spain
[c] Biomol-Informatics SL, Parque Científico de Madrid, C/ Faraday, 7, Cantoblanco, 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form 1 June 2012
Accepted 7 June 2012
Available online 15 June 2012

Keywords:
RNA degradation
RNase R
RNase II
Exosome
Temperature
Archaea

ABSTRACT

Background: Little is known regarding mRNA degradation mechanisms in archaea. In some of these single-cell organisms the existence of a complex of exoribonucleases called the exosome has been demonstrated. However, in halophilic archaea the RNase R homologue is essential since it is the only enzyme described with exoribonucleolytic activity.
Methods: In this work we have characterized the mechanism of action of *Haloferax volcanii* RNase R and its implications for the RNA degradation process. We have determined the salt, pH and divalent ion preference, and set the best conditions for the activity assays. Furthermore, we have determined the activity of the protein at different temperatures using different substrates. The dissociation constants were also calculated by Surface Plasmon Resonance. Finally, we have built a model and compared it with the *Escherichia coli* counterparts.
Results: The results obtained showed that at 37 °C, in spite of being named RNase R, this protein behaves like an RNase II protein, halting when it reaches secondary structures, and releasing a 4 nt end-product. However, at 42 °C, the optimum temperature of growth, this protein is able to degrade secondary structures, acting like RNase R.
General significance: This discovery has a great impact for RNA degradation, since this is the first case reported where a single enzyme has two different exoribonucleolytic activities according to the temperature. Furthermore, the results obtained are very important to help to decipher the RNA degradation mechanisms in *H. volcanii*, since RNase R is the only exoribonuclease involved in this process.

1. Introduction

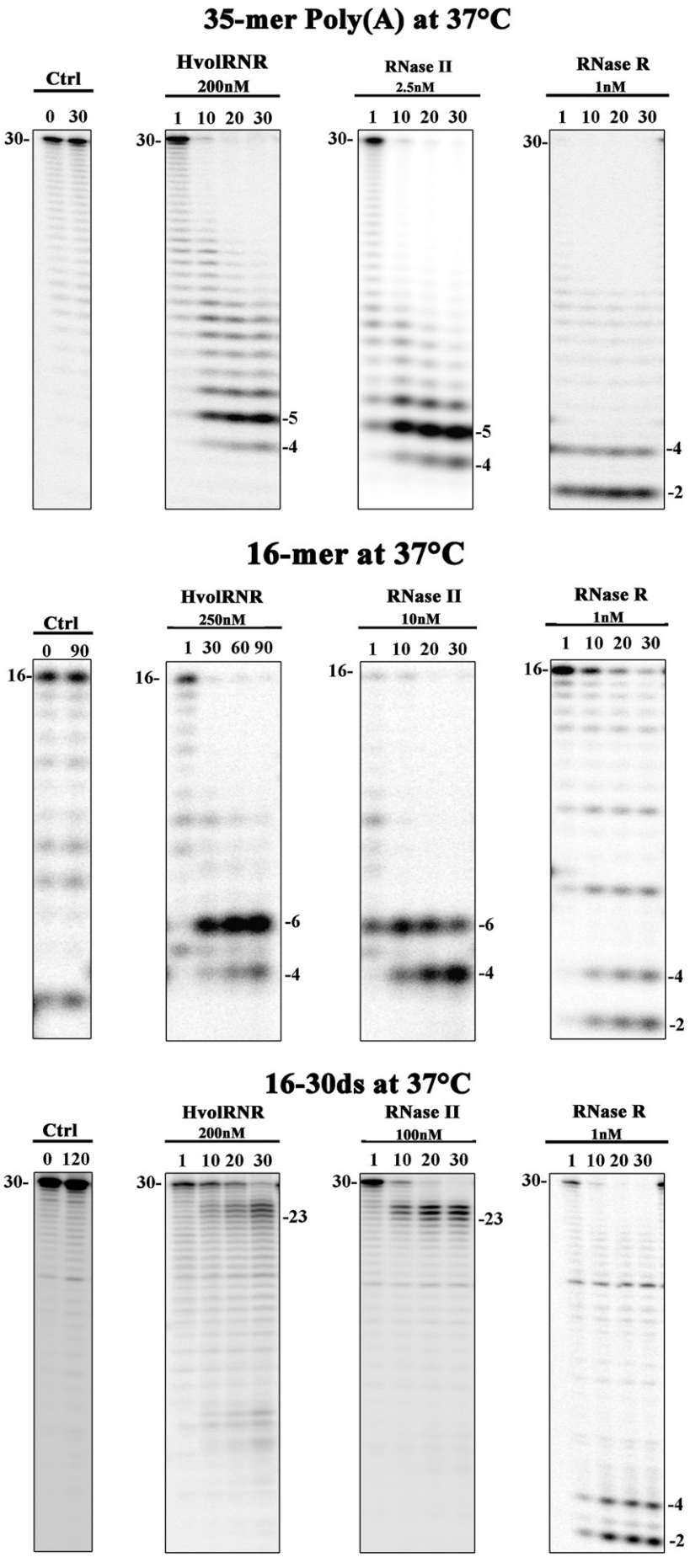
Archaea are microscopic, single-celled organisms with no nucleus, no mitochondria and no chloroplasts. Regarding mRNA, they also seem to be more similar to bacteria than to eukaryotes: they have a polycistronic mRNA with no introns, which is not modified and does not have long stabilizing poly(A) tails at the 3′ end [1]. However, little is known regarding RNA degradation in these organisms.

In some archaeal organisms, such as *Sulfolobus*, *Pyrococcus* and *Methanothermobacter*, the existence of an archaeal exosome has been demonstrated, which shares some characteristics with the eukaryotic exosome [2–5]. In both *Sulfolobus solfataricus* and *Pyrococcus abyssi* it has been shown that the exosome exhibit RNA-binding activity and has a dual function: it can work as an RNA-tailing and RNA-degrading complex, since it has both phosphorolytic and polyadenylating activity [4,6]. Halophilic and many methanogenic archaeal genomes lack the genes for the orthologues of exosomal subunits and also for a poly(A) polymerase homologue. However, an RNase R-like protein is present and it may be the only enzyme responsible for the exoribonucleolytic activity in these organisms [7]. This information indicates that different RNA degradation mechanisms may exist in archaeal organisms: one in thermophiles performed by the archaeal exosome, which involves polyadenylation, and the other in halophiles performed by other ribonucleases in the cell, in a poly(A)-independent mechanism [8]. RNA degradation in bacteria usually initiates with an endonucleolytic cleavage, followed by the cleavage of the resultant fragments by exoribonucleases, with or without a polyadenylation step [9]. In Archaea it seems that the mechanism is very similar, although some have an exosome complex, which is similar to the eukaryotic system. Most of the archaeal organisms have genes related to RNase E and RNase J [10,11], which can be responsible for the initial endonucleolytic cleavage in RNA degradation process. Then, depending on the organisms, the resultant fragments

Abbreviations: RNase, ribonuclease; CSD, Cold Shock Domain; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; SPR, Surface plasmon resonance; PDB, protein data bank; PNPase, Polynucleotide phosphorylase; PAP I, Poly(A) Polymerase; RMSD, Root mean square deviation

* Corresponding author. Tel.: +351 214469547; fax: +351 214469549.
E-mail address: cecilia@itqb.unl.pt (C.M. Arraiano).

0304-4165/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2012.06.009


35-mer Poly(A) at 37°C
Ctrl
0 30
30-
HvolRNR
200nM
1 10 20 30
30-
-5
-4
RNase II
2.5nM
1 10 20 30
30-
-5
-4
RNase R
1nM
1 10 20 30
30-
-4
-2
16-mer at 37°C
Ctrl
0 90
16-
HvolRNR
250nM
1 30 60 90
16-
-6
-4
RNase II
10nM
1 10 20 30
16-
-6
-4
RNase R
1nM
1 10 20 30
16-
-4
-2
16-30ds at 37°C
Ctrl
0 120
30-
HvolRNR
200nM
1 10 20 30
30-
-23
RNase II
100nM
1 10 20 30
30-
-23
RNase R
1nM
1 10 20 30
30-
-4
-2

are degraded in a Poly(A)-dependent manner by the archaeal exosome in thermophiles or, in the case of halophilic organisms without RNA polyadenylation, the degradation is performed by the RNase R homologue [6].

Haloferax volcanii, a microorganism isolated from the Dead Sea, is the model organism from halophilic archaea. It requires a high concentration of sodium chloride to replicate (1.7 to 2.5 M), its optimum temperature for growth is 42 °C, and it was the first organism described that is able to degrade RNA molecules in the absence of polyadenylation [6]. As an halophilic organism, *H. volcanii* does not have an exosome complex, but instead an RNase R-like protein which is essential for viability, and is probably the major enzyme involved in RNA degradation [6,12,13]. RNase R belongs to the RNase II-family of proteins, which are present in all domains of life and play a crucial role in RNA metabolism [7,9,14,15]. In eukaryotes, the RNase II homologue (called Rrp44 or Dis3) is the only active subunit of the exosome. In prokaryotes, proteins of this family are important for growth and stress responses and may also be involved in virulence [16]. RNase II, the prototype of RNase II-family of enzymes, is composed of two N-terminal CSD domains and a C-terminal S1 domain involved in RNA binding, and a central RNB domain responsible for the catalytic activity of the protein [17–19]. As a member of the RNase II-family of enzymes, RNase R shares the same domain organization with RNase II and also an overall structural rearrangement, with all the important residues for cleavage located in the same spatial position [20]. In *Escherichia coli* RNase II, Tyr253 was shown to be important for setting the final end-product of RNase II, and the same was observed with the equivalent Tyr in RNase R [20,21]. In the active site, there are four highly conserved aspartates with different roles in RNA degradation mechanism. The most critical aspartate for the activity of the enzyme is the one located in position 209 in RNase II [19,22]. Its substitution by an asparagine in RNase II, RNase R or Rrp44 (the yeast homologue) completely abolished the activity of the protein without changing its binding ability [20,21,23]. These results indicate that the mechanism of RNA degradation may be conserved in all members of this family. However, while RNase II is sensitive to secondary structures, RNase R is able to completely degrade structured substrates [14,24]. Although the crystal structure of RNase R is yet unknown, the mechanism of dsRNA degradation by RNase R has been deciphered by biochemical studies, and a model has been proposed [20,21,25–27]. It has been shown that the RNB domain of RNase R, by itself, is responsible for the degradation of double-stranded substrates [21,26], and that the RNA-binding domains are important for discriminating the molecules to be degraded, choosing only the RNAs that are tagged to be degraded [21]. More recently, it has been shown that the S1 domain from RNase R, namely the lysine-rich region (which is absent in RNase II), is also involved in the degradation of structured substrates [25]. This region was also proven to be involved in the recruitment of RNase R to stalled ribosomes and to the selective decay of defective transcripts [28].

In *H. volcanii*, the RNase R homologue is the only enzyme described with exoribonucleolytic activity, which indicates that it may play a crucial role in the mechanism of RNA degradation in this organism. The aim of this work was the characterization of the mechanism of action of RNase R from *H. volcanii* (from now on called HvolRNR) and their implications for the RNA degradation process. The results obtained showed that, although named RNase R, at 37 °C, this protein behaves like an RNase II protein: it released a 4 nt fragment as final product and is sensitive to secondary structures. However, at 42 °C, the optimum temperature of growthof *H. volcanii*, this protein is able to overcome secondary structures, acting like RNase R. This discovery has a great impact for RNA degradation, since this is the first case reported where a single enzyme has two different exoribonucleolytic activities according to temperature.

Table 1
Kinetic parameters of RNase R protein from *H. volcanii*. The kinetic constants were determined by Surface Plasmon Resonance using Biacore 2000 with a 25 nt RNA oligomer (5′-Biotin-CCCGACACCAACCACUAAAAAAAAA-3′) and 30 nt poly(A) as substrates.

	k_a (1/Ms)	k_d (1/s)	K_D (nM)
PolyA	6.2 ± 0.2 *E*03	2.5 ± 0.1 *E*−05	4.1 ± 0.1
25ss	9.4 ± 0.6 *E*04	2.5 ± 0.4 *E*−04	3.0 ± 0.4
16-25ds	6.6 ± 0.7 *E*03	1.3 ± 0.1 *E*−05	2.8 ± 0.6

2. Materials and methods

2.1. Overexpression and purification of recombinant RNase R from H. volcanii

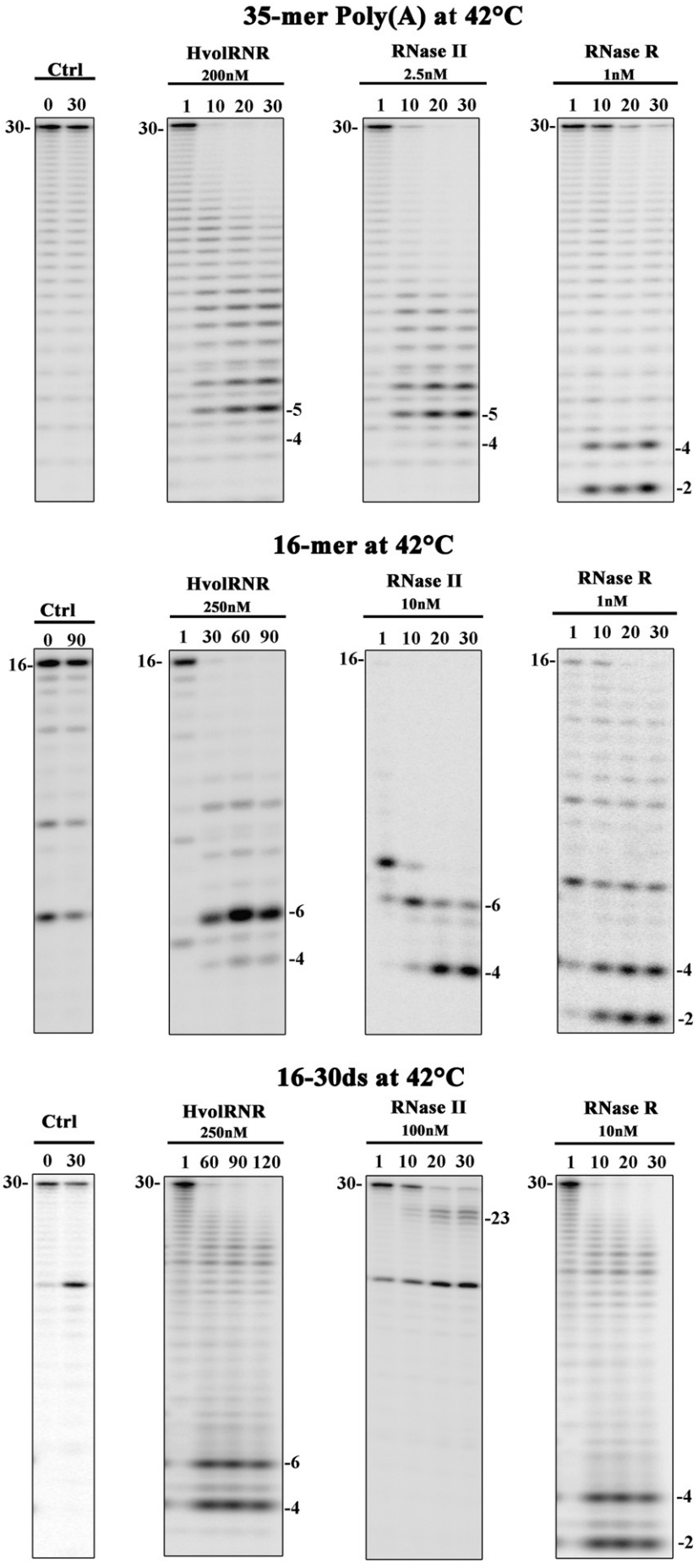
The plasmid used for expression of HvolRNR was previously described [12]. The plasmid was transformed into M15 (REP4) strain to allow the expression of the recombinant protein. Cells were grown at 37 °C in 100 ml LB medium supplemented with 150 μg/ml ampicillin to an A_{600} of 0.5 and induced by addition of 0.5 mM IPTG during 2 h. Cell culture was pelleted by centrifugation and stored at −80 °C. RNase II and RNase R overexpression and purification were performed as described previously [22,29,30].

Purification was performed by histidine affinity chromatography using HiTrap Chelating HP columns (GE Healthcare) and AKTA HLPC system (GE Healthcare) following the protocol previously described [22,30]. The fractions containing the purified HvolRNR protein were pooled together to perform an anion exchange by injecting the fractions in a monoQ column (GE Healthcare) equilibrated in buffer composed by 20 mM Tris pH 8, 60 mM KCl, 2 mM $MgCl_2$ and 0.2 mM EDTA. Protein elution was achieved by a continuous KCl gradient (from 60 mM to 1 M) in buffer B. Protein concentration was determined by spectrophotometry and 50% (v/v) glycerol was added to the final fractions prior storage at −20 °C. 0.5 μg of the purified protein was applied in a 8% SDS-PAGE and visualized by Coomassie blue staining (data not shown).

2.2. In vitro transcription

malEF RNA molecule was obtained by *in vitro* transcription using as template the pCH77 plasmid linearized with EcoRI [31]. The transcription reaction was performed using the "Riboprobe *in vitro* Transcription System" (Promega) and T7 RNA polymerase following the instructions given by manufacturers, in a 30 μl volume, containing 20 μCi of [α-32P] dUTP. The radioactively labelled RNA transcript was purified by electrophoresis on a 6% PAA/7 M urea gel. The gel slice containing the transcript was crushed and the RNA eluted overnight at room temperature with a buffer containing 0.5 M ammonium acetate pH 5.2, 1 mM EDTA, 2.5% (v/v) phenol pH 4.3. The RNA was precipitated with ethanol and resuspended in RNase free water.

Fig. 1. Exoribonucleolytic activity of HvolRNR at 37 °C: comparison with *E. coli* RNase II and RNase R. Activity assays were performed at 37 °C as described in materials and methods using three different substrates: 30-mer poly(A), the 16-mer and the double-stranded substrate 16-30ds, as referred. The concentration of proteins used is indicated in the figure. Samples were taken during the reaction at the time points indicated and reaction products were analyzed in a 20% polyacrylamide/7 M urea gel. Control reactions with no enzyme added (*Ctrl*) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated.


35-mer Poly(A) at 42°C
Ctrl
0 30
30-
HvolRNR
200nM
1 10 20 30
30-
-5
-4
RNase II
2.5nM
1 10 20 30
30-
-5
-4
RNase R
1nM
1 10 20 30
30-
-4
-2
16-mer at 42°C
Ctrl
0 90
16-
HvolRNR
250nM
1 30 60 90
16-
-6
-4
RNase II
10nM
1 10 20 30
16-
-6
-4
RNase R
1nM
1 10 20 30
16-
-4
-2
16-30ds at 42°C
Ctrl
0 30
30-
HvolRNR
250nM
1 60 90 120
30-
-6
-4
RNase II
100nM
1 10 20 30
30-
-23
RNase R
10nM
1 10 20 30
30-
-4
-2

2.3. Exoribonucleolytic activity assays

The exoribonucleolytic activity was determined using different substrates: a Poly(A) oligomer of 30 nts, a 16mer oligoribonucleotide (5′-CCCGACACCAACCACU-3′), a 30mer oligoribonucleotide (5′-CCCGACACCAACCACUAAAAAAAAAAAAAA-3′) hybridized to the complementary non-labelled 16mer oligodeoxyribonucleotide (5′-AGTGGTTGGTGTCGGG-3′), in order to obtain the double stranded substrate 16–30ds (the hybridization was performed in a 1:1 (mol:mol) ratio by 5 min of incubation at 100 °C followed by 45 min at 37 °C), and the *malEF* transcript. All the synthetic RNA molecules were labelled at its 5′ end with [γ-^{32}ATP] and T4 polynucleotide kinase. The RNA oligomers were then purified using Microcon YM-3 Centrifugal Filter Devices (Millipore) to remove the non-incorporated nucleotide. The exoribonucleolytic reactions were carried out in a final volume of 12.5 μl containing 5 nM of substrate, 20 mM Tris–HCl (pH tested from 6.5 to 9), KCl or NaCl (from 10 to 200 mM), $MgCl_2$ (from 1 to 10 mM), and 1 mM DTT. The amount of each enzyme added to the reaction was adjusted to obtain linear conditions and is indicated in the figures and respective legends. Reactions were started by the addition of the enzyme and incubated at 37 °C or 42 °C. Samples were withdrawn at the time points indicated and the reaction was stopped by adding formamide-containing dye supplemented with 10 mM EDTA. Reaction products were resolved in a 20% polyacrylamide/7 M urea and detected by using the Fuji Fluorescent Analyzer TLA-5100 from GE Healthcare. The exoribonucleolytic activity of the enzyme was determined by measuring and quantifying the disappearance of the substrate in several distinct experiments in which the protein concentration was adjusted in order that, under those conditions, less than 25% of substrate was degraded. Each value obtained represents the mean for these independent assays.

2.4. Surface plasmon resonance analysis—BIACORE

Biacore SA chips were obtained from Biacore Inc. (GE Healthcare). The flow cells of the SA streptavidin sensor chip were coated with a low concentration of the following substrates. On flow cell 1 no substrate was added so this cell could be used as the control blank cell. On flow cell 2 a 5′ biotinylated 25-nucleotide RNA oligomer (5′-CCCGACACCAACCACUAAAAAAAAA-3′) was added to allow the study of the protein interaction with a single-stranded RNA molecule. On flow cell 3 a 5′ biotinylated 30-mer PolyA substrate. On flow cell 4 the biotinylated 25mer hybridized with the complementary 16mer oligodeoxyribonucleotide (5′-AGTGGTTGGTGTCGGG-3′) was immobilized, originating the double-stranded substrate 16-25ds. The target substrates were captured on flow cells 2 to 4 by manually injecting 20 μl of a 500 nM solution of the substrates in the reaction buffer at a 20 μl/min flow rate. The biosensor assay was run at 4 °C in the buffer with 20 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM DTT and 25 mM EDTA. The proteins were injected over flow cells 1, 2, 3 and 4 for 2.5 min at concentrations of 10, 20, 30, 40 and 50 nM using a flow rate of 20 μl/min. All experiments included triple injections of each protein concentration to determine the reproducibility of the signal and control injections to assess the stability of the RNA surface during the experiment. Bound protein was removed with a 30-s wash with 2 M NaCl. Data from flow cell 1 were used to correct for refractive index changes and non-specific binding. Rate and equilibrium constants were calculated using the BIA EVALUATION 3.0 software package, according to the fitting model 1:1 Languimir Binding.

2.5. Theoretical model of RNase R from H. volcanii

A complete structural model of HvolRNR wild-type protein (D4H014_HALVD) was built up by using software DeepView [32], standard comparative methods, and published crystallographic structures of RNase II proteins from *E. coli* (PDB codes: 2IX0, 2IX1 [17] and 2ID0 [19]). The structural quality of the model was checked using analysis programs (Anolea, Gromos and Verify3D) provided by the SWISS-MODEL server (http://swissmodel.expasy.org) [33–35]. A coarse model of the hypothetical interaction of modeled RNase R and one single-strand poly-A RNA molecule was performed by simple structural superposition of the available ssRNA structure in complex with RNase II D209N mutant from *E. coli* [17,19]. To optimize local geometries and contacts, the resulting model was subjected to a standard protocol of energy minimization and 200 picoseconds of molecular dynamics simulation as described elsewhere [36]. All structures were manipulated and visually rendered using DeepView and Pymol [37].

3. Results and discussion

3.1. Determining the optimal conditions for HvolRNR activity

H. volcanii is an organism that lives in Dead Sea, which has an extremely high salt concentration. In order to see if HvolRNR had adapted to be active in high salt concentrations, we analyzed the activity of the protein using two different types of salts, KCl and NaCl, in five different concentrations, 50, 100 and 500 mM and 1 and 2 M. The results obtained for KCl are in agreement with the ones previously published, showing that HvolRNR prefers lower KCl concentrations (Fig. S1a) [12]. In the presence of NaCl, the activity of the protein was highly impaired (Fig. S1a). We quantified the activity of the protein in the conditions tested. For this quantification, we adjusted the conditions of the reaction to guarantee that less than 25% of the substrate was degraded. The activity of the protein was then measured by quantifying the disappearance of the substrate over time. This determination confirmed that HvolRNR prefers lower KCl concentrations, being more active in the presence of 50 and 100 mM (Fig. S1b). For subsequent experiments, we used 100 mM of KCl. Although being an organism which lives in the Dead Sea (which has salt concentration nearing saturation), and which also has a high internal potassium concentration (~4 M), these results demonstrate that this protein did not adapt to have an optimal activity inside the cell. This led us to agree with what was previously proposed: the activity that the protein presents at high salt concentrations is sufficient for the RNA degradation activity required by *H. volcanii* [12].

Afterwards, we analyzed the effect of pH on catalysis. For this purpose, we tested HvolRNR activity using pH ranging from 6.5 to 9. RNase R accumulated higher amounts of degradation products at pH 8 (Fig. S2a). This result was confirmed when the activity of the protein was quantified, although at pH 8.5 and 9 we obtained similar results (Fig. S2b).

It had already been demonstrated that exoribonucleases from the RNase II-family of enzymes need a divalent ion in order to proceed with catalysis. For *E. coli* RNase II and RNase R, the presence of Mg^{2+} was shown to be very important for the activity of the proteins

Fig. 2. Exoribonucleolytic activity of HvolRNR at 42 °C: comparison with *E. coli* RNase II and RNase R. Activity assays were performed at 42 °C as described in materials and methods using three different substrates: 30-mer poly(A), the 16-mer and the double-stranded substrate 16-30ds, as referred. The concentration of proteins used is indicated in the figure. Samples were taken during the reaction at the time points indicated and reaction products were analyzed in a 20% polyacrylamide/7 M urea gel. Control reactions with no enzyme added (*Ctrl*) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated.

[17]. However, other divalent ions can also contribute to the activity of these proteins (namely Zn^{2+} in a 10 μM concentration for RNase R from *E. coli* and *Mycoplasma genitalium* [38]). We performed activity assays with *E. coli* RNase II and RNase R in the presence of different divalent ions: Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}. Both enzymes showed activity in the presence of divalent ions other than Mg^{2+} (Fig. S3). For *E. coli* RNase II, we were able to detect activity in the presence of Mg^{2+}, Ca^{2+}, Zn^{2+} and Ni^{2+} (the last two only with higher incubation periods). The incubation of RNase R in the same conditions showed that it was active in the presence of the same ions, however only Mg^{2+} works properly for catalysis at short incubation periods (Fig. S3b). The same experiment was performed for HvolRNR (Fig. S4a). This protein had activity in the presence of Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+}, and longer incubation periods were not necessary in order to detect activity. We determined the specific activity of HvolRNR with the different ions (Fig. S4a, right panel). The higher activity was obtained in the presence of Mg^{2+}, although we had obtained a similar activity with Ca^{2+}. We also tested different Mg^{2+} concentrations and, from the concentrations showed, 1 mM was the one that allowed a higher activity (Fig. S4b, right panel). We also tested lower Mg^{2+} concentrations and the values obtained are very similar to the ones obtained with 1 mM, and then decreased for the lowest values (data not shown).

For the following experiments, we used the reaction conditions that were optimised as described above. To prevent degradation of substrates during SPR experiments, Mg^{2+} was omitted and EDTA added to the buffer.

3.2. HvolRNR behaves like an RNase II-like protein

The aim of this work was to characterize the activity of HvolRNR. It has been shown for RNase II and RNase R from *E. coli* that the use of synthetic RNA is efficient to characterize its activity [20,21]. Taking this into consideration, we used three synthetic substrates (two single-stranded and one double-stranded) to analyze the activity of HvolRNR.

Although *E. coli* RNase II and RNase R share some catalytic properties, they are different regarding the final product released and also in their ability to degrade structured molecules [9]. RNase II degrades RNA molecules releasing a product with 4 nucleotides of length and stalls 5 to 7 nt before it reaches a double-stranded region. On the contrary, RNase R is able to overcome secondary structures and degrades the RNA molecules releasing a final product of 2 nt [14,29].

Regarding the amino acid sequence, HvolRNR is more similar to *E. coli* RNase R (34% of identity) than to RNase II (26% of identity) [12]. For this reason, we would expect that its activity would be similar to *E. coli* RNase R. However, when we analyzed the RNA degradation behaviour, the results were different than expected. For the poly(A) substrate, HvolRNR accumulated an intermediary fragment of 5 nucleotides of length, which was then converted to a 4 nt fragment as the reaction proceeded (Fig. 1). This degradation pattern is typical for RNase II protein and not RNase R (Fig. 1). With higher protein concentration and longer incubation periods, we observed that the intermediate fragment of 5 nt was completely converted into the 4 nt product and no smaller products were generated (data not shown). A similar result was obtained for the 16-mer substrate. However, the intermediate fragment observed has 6 nt of length instead of 5 nt (Fig. 1). This may be related with substrate sequence, namely with the fact that the fifth nucleotide counting from the 5´ end is an adenine. It has already been shown that proteins from the RNase II-family prefer poly(A) substrates [20,36]. However, when we measured the dissociation constants using three different substrates, the results showed that HvolRNR does not present any substrate preference (Table 1). By analysing the k_a and k_d values, we can see that for the 25ss (which has a random sequence similar to 16-mer) there are higher association and dissociation rates when compared to the other RNA molecules. The results obtained with the single-stranded substrates showed that HvolRNR behaved like an RNase II-like protein, since it released a 4 nt fragment.

Next, we wanted to check the behaviour of HvolRNR in the presence of a double-stranded substrate, the 16-30ds. This substrate was already described in the literature [18] and consists of a 30-mer chain hybridized at its 5' end with a complementary chain of 16 nucleotides of length. This substrate has, at the 3' extremity, a single-stranded tail with 14 nucleotides, which is enough to allow the attachment of the enzymes . As already mentioned, *E. coli* RNase II is sensitive to secondary structures while RNase R is able to degrade highly structured substrates. When we tested the HvolRNR activity with the 16-30ds substrate, the protein showed a similar behaviour to the *E. coli* RNase II protein, since it was not able to degrade structured molecules, stalling 7 nucleotides before it reached the double-stranded region (Fig. 1). Even when we used higher protein concentrations and a prolonged

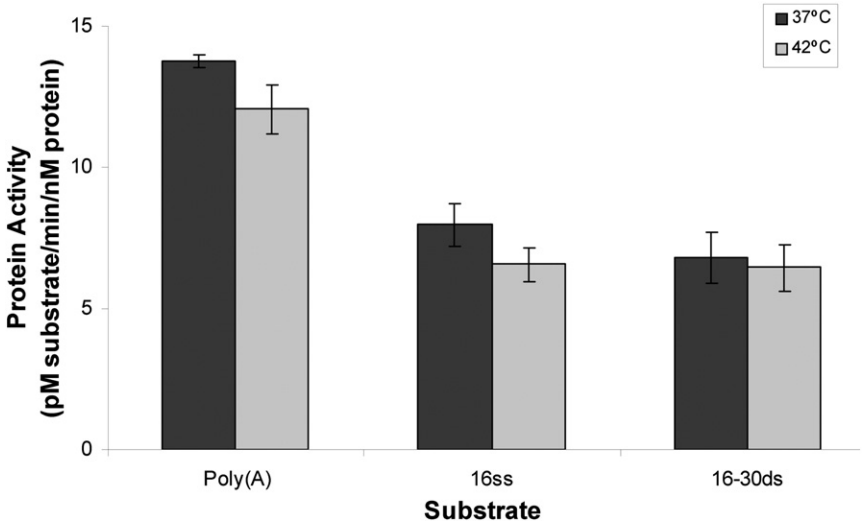


Fig. 3. Determination of the activity of HvolRNR at two different temperatures. The activity of the protein was determined at 37 °C and 42 °C using three different substrates: 30-mer poly(A), 16-mer and the double-stranded 16-30ds. All the activity assays were performed in triplicate.

incubation time the results remained unaltered (data not shown). Considering that we are in the presence of an organism which lacks polyadelylation [6], we could expect that it would compensate the degrading activity on structured RNAs. However, this was not the case since this protein was not able to degrade structured RNAs in the conditions tested. The results obtained led us to conclude that, although the *H. volcanii* homologue presented a higher similarity to *E. coli* RNase R regarding its amino acid sequence [12], it behaves like an RNase II-like protein since it is not able to degrade double-stranded substrates and releases a fragment of 4 nt as end-product of the reaction.

3.3. HvolRNR is able to degrade some double stranded substrates at 42 °C

Although *H. volcanii* is able to grow at 37 °C, its optimal temperature of growth is around 42 °C [13]. Because the results described above were all performed at 37 °C, we also decided to test the activity of the protein in the same conditions and with the same substrates at 42 °C.

The results obtained with the single-stranded substrates at 42 °C were similar to the ones obtained at 37 °C (Figs. 1 and 2). However, for the poly(A) substrate we could observe that the ability to degrade the smaller RNA fragments is reduced at 42 °C for the three proteins. For HvolRNR and *E. coli* RNase II we could observe that the same conditions used at 37 °C are not enough for the proteins to degrade the RNA molecules until the 4 nt (Fig. 2). Only when higher incubation periods were used were we able to see the final degradation products (data not shown). We determined the activity of HvolRNR at both temperatures, and, for the three substrates we could see that it was just slightly reduced at 42 °C (Fig. 3). This determination was performed taking into account the amount of substrate degraded over time and not the formation of the 4 nt end-product. Probably, this was the reason why we did not detect any significant differences regarding the activity at both temperatures.

Surprisingly, when we tested the activity of the HvolRNR against the 16-30ds at 42 °C, the result was not similar to the one obtained at 37 °C. While at 37 °C the protein was not able to degrade double-stranded substrates, behaving like RNase II, at 42° the protein was now able to overcome secondary structures and degrade the 16-30ds substrate completely releasing a mixture of fragments with 4 and 6 nt of length (Fig. 2). It is known that at high temperatures the RNA structures are less stable, which led us to ask if the result obtained could be due to a destabilization of the structure of the RNA molecule. Moreover, if we calculate the Tm of this substrate, we obtain a value of 46 °C. In order to test this hypothesis, we also tested RNase II as a negative control and RNase R as a positive control. At the same temperature we were able to see that RNase II was still not able to degrade the 16-30ds substrate, while RNase R, as expected, was able to overcome the secondary structure (Fig. 2). In RNase II gel, we could observe a band which increases in intensity during the reaction time. This band also appears in the control reaction and is unspecific. This experiment allowed us to verify the integrity of our structured RNA at this temperature, since RNase II was not able to degrade it.

Considering the results obtained at 42 °C with the 16-30ds substrate, we decided to perform another experiment, but this time using an mRNA transcript corresponding to the intergenic region of the malE–malF operon. This transcript has 375 nucleotides and two stem-loop structures at the 3′ end: a large secondary structure formed by the two inverted palyndromic REP sequences and a smaller and weaker secondary structure of the mRNA [31,39]. The *E. coli* RNase II enzyme rendered two main intermediate products (P1 and P2), which is in agreement with previously published results [22,31] (Fig. 4). Such intermediates correspond to the stalling of the enzyme in the vicinity of the two secondary structures of the mRNA. Considering that the first secondary structure at the 3′ end of the transcript

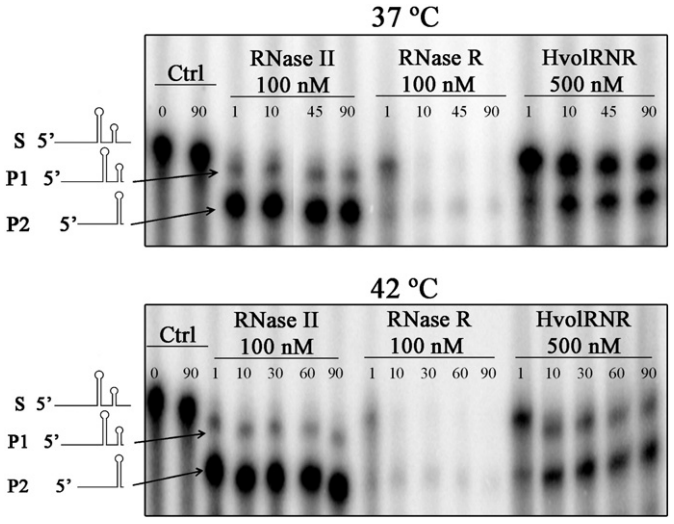


Fig. 4. Exoribonucleolytic activity of HvolRNR using the malE–malF transcript: comparison with *E. coli* RNase II and RNase R. Activity assays were performed at 37 °C (top panel) and 42 °C (bottom panel) as indicated in materials and methods using the malE–malF substrate. The concentration of proteins used is indicated in the figure. Samples were taken during the reaction at the time points indicated and reaction products were analyzed in a 5% polyacrylamide/7 M urea gel. Control reactions with no enzyme added (*Ctrl*) were incubated at the maximum reaction time for each protein. S—substrate; P1 and P2—intermediary degradation products.

is weak (Tm of ~30 °C), RNase II can easily degrade it (P1). However, it stalls in the vicinity of the second secondary structure, which is more stable (P2) and has a Tm higher than 70 °C (Fig. 4). No alterations were observed when the temperature of the reaction was increased from 37 °C to 42 °C. The results obtained for *E. coli* RNase R showed that this enzyme completely degraded the malE–malF substrate at both temperatures. The products of the reaction are not observed in this 5% PAA gel, due to the different sizes of final products between RNase II and RNase R. Regarding the activity of HvolRNR protein at 37 °C, we could observe that the major product of degradation is P1. This means that this protein has difficulties at 37 °C in degrading secondary structures even those that are not very stable and that are easily degraded by *E. coli* RNase II (Fig. 4). However, at 42 °C the main product of degradation is the P2. Also, considering the differences in band intensity between RNase II and HvolRNR at these temperature and the fact that the assays were performed at the same time with the same amount of substrate, we cannot exclude the possibility that some substrate was completely degraded (Fig. 4). It was previously reported that thermal breathing plays a role in the degradation of dsRNA by *E. coli* RNase R [27]. The results presented here also indicate that the degradation of structured substrates by HvolRNR depends on temperature, which suggests that the thermal breathing is also important for the mechanism of action of this protein.

These results led us to build a model for RNase R from *H. volcanii*. Multiple sequence alignment of the *Haloferax* protein to other RNases from bacteria [20] reveals the lack of the characteristic RNA-binding domains CSD1, CSD2 and S1 with the lysine-rich motifs. Interestingly, the RNase from *Haloferax* would have one recognizable RNB domain within its constitution, showing some of the residues deemed to be critical for the full conservation of catalytic and processing functions in bacterial RNases (Fig. 5b). This hypothetically conserved constitutive RNB domain in *Haloferax* would be shorter and flanked by two amino and carboxy-terminal regions, with lengths of 30 and 28 residues respectively, and with very low sequence identity similarity to their counterparts in other RNases (Fig. 5a). The rendered three-dimensional model for *Haloferax* protein predicts a structural architecture fully compatible with the characteristic single alpha/beta-fold of RNB domains (averaged RMSD to templates of 0.93 Å).

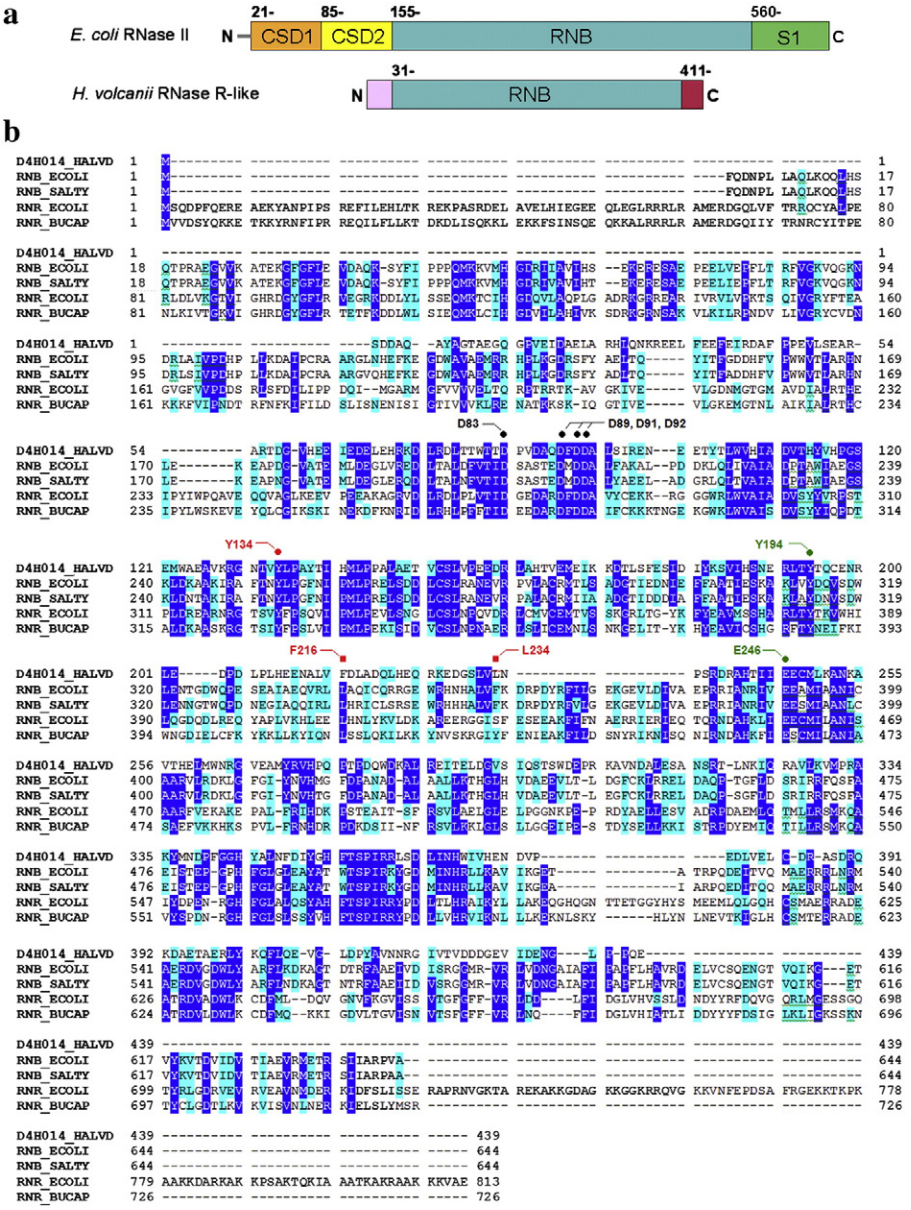


Fig. 5. a. Linear representations of the domain organization of the RNase II real structure from PDB code: 2IX0 [2] in comparison to *H. volcanii* RNase-R like protein: RNB domain in blue color and terminal flanking regions in pink and dark red respectively. **b.** Structure-based sequence alignment of *H. volcanii* (UniProt code D4H014_HALVD) and *E. coli* (UniProt code RNB_ECOLI) proteins. Both sequences are coloured according to conservation. Positions of some functional residues are highlighted with black dots for Asp83, Asp89, Asp91 and Asp 92 (critical/important for catalysis), blue dot for Tyr194 (RNA clamping) and red dots for Tyr134 and Phe216 (RNA specificity) in the RNB domain from *H. volcanii*. Situation of Leu234 (position possibly correlated to Phe216) also highlighted with a red square.

Examination of its putative interaction model with one single-strand poly-A RNA chain (Fig. 6) shows complete absence of structural collisions between the protein residues and the stacked nucleotides and magnesium ion.

Further analyses on the structural location in the theoretical model of critical and important conserved residues in other RNases also support the homology hypothesis (Figs. 5 and 6). Due to their close vicinity to the position of the magnesium atom and the outgoing 3′-OH nucleotide (nt1) in the modeled active centre, Asp83, Asp89, Asp91 and Asp92 in *Haloferax* could perform similar roles in the catalytic mechanism as their equivalents in RNase II (Asp201, Asp207, Asp209 and Asp210) [20,22] and RNase R (Asp272, Asp278, Asp280 and Asp281) [21,40] from *E. coli*. Regarding the discrimination for RNA substrates by recognition of ribose moieties, the location of residues Tyr194 and Glu246 in *Haloferax* model suggests that functional equivalence might exist also with their homologous residues in RNase II (Tyr313 and Glu390) from *E. coli* [36], and hypothetically in RNase R. Finally, upon the same structural and evolutionary considerations, Tyr134 in *Haloferax* RNase-like protein could be involved in setting the length of final products, as residues Tyr253 in RNase II [20] and Tyr324 in RNase R [21] from *E. coli* perform alike. Regarding other correspondences to residues responsible for substrate binding, the position of Phe358 (nt5 clamping) in the RNase II from *E. coli* is held in *H. volcanii* by Leu234, which is located beside the catalytic groove in the structural model. Exceptionally, *Haloferax* protein shows one phenylalanine residue (Phe216), whose corresponding residue in RNase II from *E. coli* is Leu340, in close proximity to nucleotides nt4–nt5 in the theoretical structure. Although it is not feasible to infer from this model the precise interaction of Phe216 to the RNA, its hypothetical location suggests a common functional role with other aromatic residues in RNase II and RNase R, namely as molecular clamps involved in partially setting the end-product length in these families. Such correlated and punctual changes in

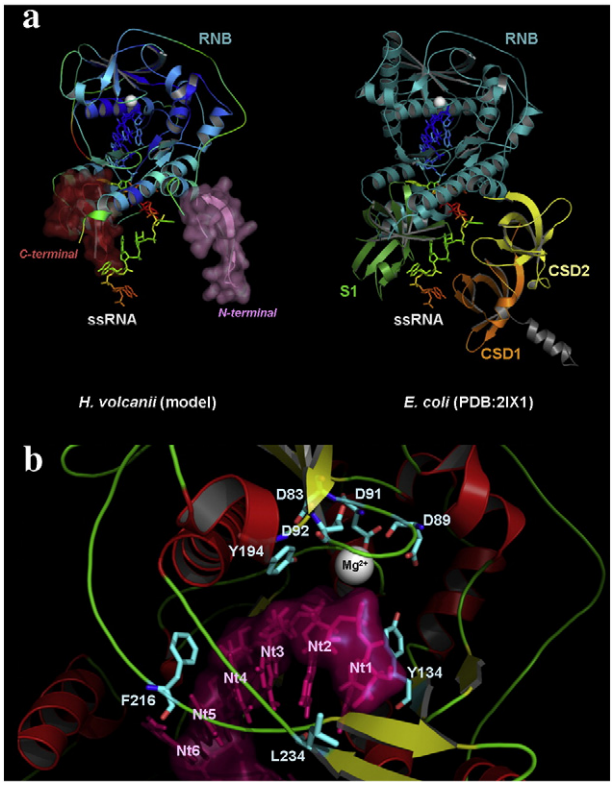


Fig. 6. a. Left. Theoretical 3D model of RNase R-like protein from *H. volcanii* in complex with one single-strand RNA poly-A (ssRNA) and Mg^{2+} (white sphere). Predicted structure for the RNB domain coloured in a blue-to-red gradation according to high-to-low confidence scores provided by the modeling procedures. Low-confidence modeled structures corresponding to the N-terminal and C-terminal flanking regions of *H. volcanii* are depicted as van-der-Waals surfaces in pink and dark red color respectively. Right. Depicted for comparison purposes only, real structure of the RNase II D209N mutant from E. coli (PDB code 2IX1) [2] showing the relative position of RNB, CSD1, CSD2 and S1 domains to RNase R-like protein from *H. volcanii*. (Common ssRNA structure colored as the original B-factors from from Frazão et al. [2]). **b**. Insight into the model of *Haloferax* RNase R active center within RNB domain and its catalytic groove. Relative positions and side chains of important or functionally relevant residues (Asp83, Asp89, Asp91, Asp 92, Tyr134, Tyr194, Phe216) are shown in sticks and coloured by atom type. Predicted secondary structure elements colored in red (alpha), yellow (beta) and green (coil). Hypothetical position of the magnesium ion and nucleotides Nt1 to Nt6 from ssRNA within the catalytic channel depicted in magenta sticks and van-der-Waals surface (sugar-phosphate backbone).

the region could have been obtained by hypothetical mutual variations among sites, possibly related to the structural reorganization of the whole binding interface throughout evolution.

Experimental results and structural insights on theoretical models strongly support the hypothesis that the protein from *H. volcanii* holds a shorter but fully functional RNB domain, structurally homologous to those in bacteria and eukaryotes. Nevertheless, characteristic companion domains (as S1 or CSD) present in other RNase proteins might be missing in the real structure of *H. volcanii* given the absence of homologous amino- or carboxy-terminal counterparts in its sequence (Fig. 5a). Although modeling procedures also rendered three-dimensional structures compatible for those regions, those cannot be supported by any sequence-based homology relationship. Consequently, the real structure of these terminal regions in *H. volcanii* RNase R is very uncertain. Although a number of experimental studies have shown that nuclease domain alone in RNase R proteins is sufficient to bind and degrade structured RNAs [21,26], it cannot be discarded that, in the case of *H. volcanii* RNase R, the amino and carboxi-terminal flanking regions could be implicated in critical or sideline RNA helicase functions. Moreover, and according to the experimentally assessed dual behaviour at different temperature ranges, short RNB-flanking regions in the real structure might be partially mimicking CSD and S1 roles.

3.4. Conclusions

In *H. volcanii* there is no archaeal exosome and its genome lacks a number of proteins involved in RNA degradation, namely PNPase and PAP I. Only one gene for a putative exoribonuclease is found and it has been shown to have significant homology to RNase R. This protein is essential for cell survival, which indicates that it plays a crucial role in RNA metabolism and it may be the only protein responsible for the exoribonucleolytic activity observed in *H. volcanii* [6,12]. By analysing the activity of this protein, we could see that, although it is more similar in its amino acid sequence to RNase R [12], at 37 °C it behaves like RNase II and it is not able to degrade structured RNAs. At high salt concentrations, RNA is encouraged to become more structured. We can speculate how *H. volcanii*, which has a high internal salt concentration, is able to deal with RNA degradation in the absence of a protein which is able to overcome double-stranded substrates. One possible explanation is that the RNA degradation could be performed mainly by endoribonucleases which would cleave all double-stranded regions and leave only single-stranded substrates to be degraded by RNase R. However, in *H. volcanii* genome we can only find homologues to RNase Z, RNase H and RNase P. RNase Z have already been described as being involved in tRNA processing and, more recently, in the maturation of 5S rRNA [41,42]. The activity of RNase H and RNase P from *H. volcanii* has not yet been characterized but they are involved in the degradation of the RNA of RNA/DNA hybrids that are formed during replication and repair, or in the maturation of the 5' ends of tRNAs, respectively [9].

However, at 42 °C, the optimal temperature of growth, we observe a different scenario. At this temperature, we have shown that HvolRNR is able to degrade the synthetic 16-30ds substrate. Moreover, the degradation of the malE–malF transcript is altered at this temperature. At 37 °C HvolRNR had difficulties in degrading even less stable secondary structures that are easily degraded by *E. coli* RNase II. However, at 42 °C the degradation of these structures was more efficient. These results indicate that the degradation of structured substrates by HvolRNR depends on the temperature, which suggests that the thermal breathing is also important for the mechanism of action of this protein.

This work characterized the HvolRNR protein from *H. volcanii*. Although it was predicted to be more similar to *E. coli* RNase R, the results presented here show that at 37 °C, it behaves like RNase II regarding the ability to degrade double stranded substrates and also in the final product released. Surprisingly, when we tested the activity of the protein at 42 °C, we could see that it was able to degrade dsRNAs. We are therefore in the presence of a protein with dual activity, which can act like RNase II or RNase R, according to the temperature. The results obtained are very important in helping to decipher the RNA degradation mechanisms in *H. volcanii*, since RNase R is the only exoribonuclease involved in this process.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2012.06.009.

Acknowledgments

Rute G. Matos was a recipient of a PhD fellowship funded by FCT-Fundação para a Ciência e a Tecnologia, Portugal. We thank Andreia Aires for technical support in the lab. She was a recipient of a BTI grant also from FCT. We acknowledge Prof. Gadi Schuster for the

plasmid overexpressing *H. volcanii* RNase R. We thank Edward Zungailia for reading the manuscript. We thank Centro de Computación Científica-UAM for computational support. Work at CBMSO was supported by fellowships and grants from the Spanish Ministerio de Ciencia e Innovación (SAF2007-61926, IPT2011-0964-900000 and SAF2011-13156-E) and the European Commission (FP7 HEALTH-F3-2009-223431). Biomol-Informatics was financed by the European Social Fund. Support from the "Fundación Ramón Areces" is acknowledged. Work at ITQB was supported by grants from Fundação para a Ciência e a Tecnologia, including grant PEst-OE/EQB/LA0004/2011, also from FCT.

References

[1] J.W. Brown, J.N. Reeve, Polyadenylated RNA isolated from the archaebacterium *Halobacterium halobium*, J. Bacteriol. 166 (1986) 686–688.
[2] E. Evguenieva-Hackenberg, P. Walter, E. Hochleitner, F. Lottspeich, G. Klug, An exosome-like complex in *Sulfolobus solfataricus*, EMBO Rep. 4 (2003) 889–893.
[3] M.H. Farhoud, H.J. Wessels, P.J. Steenbakkers, S. Mattijssen, R.A. Wevers, B.G. van Engelen, M.S. Jetten, J.A. Smeitink, L.P. van den Heuvel, J.T. Keltjens, Protein complexes in the archaeon *Methanothermobacter thermautotrophicus* analyzed by blue native/SDS-PAGE and mass spectrometry, Mol. Cell. Proteomics 4 (2005) 1653–1663.
[4] C.R. Ramos, C.L. Oliveira, I.L. Torriani, C.C. Oliveira, The *Pyrococcus* exosome complex: structural and functional characterization, J. Biol. Chem. 281 (2006) 6751–6759.
[5] M.V. Navarro, C.C. Oliveira, N.I. Zanchin, B.G. Guimaraes, Insights into the mechanism of progressive RNA degradation by the archaeal exosome, J. Biol. Chem. 283 (2008) 14120–14131.
[6] V. Portnoy, E. Evguenieva-Hackenberg, F. Klein, P. Walter, E. Lorentzen, G. Klug, G. Schuster, RNA polyadenylation in Archaea: not observed in *Haloferax* while the exosome polynucleotidylates RNA in *Sulfolobus*, EMBO Rep. 6 (2005) 1188–1193.
[7] Y. Zuo, M.P. Deutscher, Exoribonuclease superfamilies: structural analysis and phylogenetic distribution, Nucleic Acids Res. 29 (2001) 1017–1026.
[8] E.V. Koonin, Y.I. Wolf, L. Aravind, Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach, Genome Res. 11 (2001) 240–252.
[9] C.M. Arraiano, J.M. Andrade, S. Domingues, I.B. Guinote, M. Malecki, R.G. Matos, R.N. Moreira, V. Pobre, F.P. Reis, M. Saramago, I.J. Silva, S.C. Viegas, The critical role of RNA processing and degradation in the control of gene expression, FEMS Microbiol. Rev. 34 (2010) 883–923.
[10] V. Anantharaman, E.V. Koonin, L. Aravind, Comparative genomics and evolution of proteins involved in RNA metabolism, Nucleic Acids Res. 30 (2002) 1427–1464.
[11] S. Even, O. Pellegrini, L. Zig, V. Labas, J. Vinh, D. Brechemmier-Baey, H. Putzer, Ribonucleases J1 and J2: two novel endoribonucleases in *B.subtilis* with functional homology to *E.coli* RNase E, Nucleic Acids Res. 33 (2005) 2141–2152.
[12] V. Portnoy, G. Schuster, RNA polyadenylation and degradation in different Archaea; roles of the exosome and RNase R, Nucleic Acids Res. 34 (2006) 5923–5931.
[13] M.F. Mullakhanbhai, H. Larsen, *Halobacterium volcanii* spec. nov., a Dead Sea *Halobacterium* with a moderate salt requirement, Arch. Microbiol. 104 (1975) 207–214.
[14] J.M. Andrade, V. Pobre, I.J. Silva, S. Domingues, C.M. Arraiano, The role of 3′–5′ exoribonucleases in RNA degradation, Prog. Mol. Biol. Transl. Sci. 85 (2009) 187–229.
[15] R.G. Matos, V. Pobre, F.P. Reis, M. Malecki, J.M. Andrade, C.M. Arraiano, Struture and degradation mechanisms of 3' to 5' exoribonucleases, ribonucleases, Nucleic Acids Mol. Biol. 26 (2011) 193–222.
[16] C.M. Arraiano, R.G. Matos, A. Barbas, RNase II: the finer details of the Modus operandi of a molecular killer, RNA Biol. 7 (2010) 276–278.
[17] C.E. McVey, M. Amblar, A. Barbas, F. Cairrao, R. Coelho, C. Romao, C.M. Arraiano, M.A. Carrondo, C. Frazao, Expression, purification, crystallization and preliminary diffraction data characterization of *Escherichia coli* ribonuclease II (RNase II), Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun. 62 (2006) 684–687.
[18] M. Amblar, A. Barbas, A.M. Fialho, C.M. Arraiano, Characterization of the functional domains of *Escherichia coli* RNase II, J. Mol. Biol. 360 (2006) 921–933.
[19] Y. Zuo, H.A. Vincent, J. Zhang, Y. Wang, M.P. Deutscher, A. Malhotra, Structural basis for processivity and single-strand specificity of RNase II, Mol. Cell 24 (2006) 149–156.
[20] A. Barbas, R.G. Matos, M. Amblar, E. Lopez-Viñas, P. Gomez-Puertas, C.M. Arraiano, New insights into the mechanism of RNA degradation by ribonuclease II: identification of the residue responsible for setting the RNase II end product, J. Biol. Chem. 283 (2008) 13070–13076.
[21] R.G. Matos, A. Barbas, C.M. Arraiano, RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation, Biochem. J. 423 (2009) 291–301.
[22] M. Amblar, C.M. Arraiano, A single mutation in *Escherichia coli* ribonuclease II inactivates the enzyme without affecting RNA binding, FEBS J. 272 (2005) 363–374.
[23] A. Dziembowski, E. Lorentzen, E. Conti, B. Seraphin, A single subunit, Dis3, is essentially responsible for yeast exosome core activity, Nat. Struct. Mol. Biol. 14 (2007) 15–22.
[24] H.A. Vincent, M.P. Deutscher, Substrate recognition and catalysis by the exoribonuclease RNase R, J. Biol. Chem. 281 (2006) 29769–29775.
[25] R.G. Matos, A. Barbas, P. Goméz-Puertas, C.M. Arraiano, Swapping the domains of exoribonucleases RNase II and RNase R: conferring upon RNase II the ability to degrade dsRNA, Proteins 79 (2011) 1853–1867.
[26] H.A. Vincent, M.P. Deutscher, 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. 284 (2009) 486–494.
[27] H.A. Vincent, M.P. Deutscher, Insights into how RNase R degrades structured RNA: analysis of the nuclease domain, J. Mol. Biol. 387 (2009) 570–583.
[28] Z. Ge, P. Mehta, J. Richards, A.W. Karzai, Non-stop mRNA decay initiates at the ribosome, Mol. Microbiol. 78 (2010) 1159–1170.
[29] M. Amblar, A. Barbas, P. Gomez-Puertas, C.M. Arraiano, The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization, RNA 13 (2007) 317–327.
[30] C.M. Arraiano, A. Barbas, M. Amblar, Characterizing ribonucleases *in vitro*: examples of synergies between biochemical and structural analysis, Methods Enzymol. 447 (2008) 131–160.
[31] R.S. McLaren, S.F. Newbury, G.S. Dance, H.C. Causton, C.F. Higgins, mRNA degradation by processive 3'-5' exoribonucleases in vitro and the implications for prokaryotic mRNA decay in vivo, J. Mol. Biol. 221 (1991) 81–95.
[32] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, Electrophoresis 18 (1997) 2714–2723.
[33] K. Arnold, F. Kiefer, J. Kopp, J.N. Battey, M. Podvinec, J.D. Westbrook, H.M. Berman, L. Bordoli, T. Schwede, The protein model portal, J. Struct. Funct. Genomics 10 (2009) 1–8.
[34] F. Kiefer, K. Arnold, M. Kunzli, L. Bordoli, T. Schwede, The SWISS-MODEL repository and associated resources, Nucleic Acids Res. 37 (2009) D387–D392.
[35] M.C. Peitsch, Protein modeling by E-mail, Bio/Technology 13 (1995) 658–660.
[36] A. Barbas, R.G. Matos, M. Amblar, E. Lopez-Vinas, P. Gomez-Puertas, C.M. Arraiano, Determination of key residues for catalysis and RNA cleavage specificity: one mutation turns RNase II into a "super-enzyme", J. Biol. Chem. 284 (2009) 20486–20498.
[37] W.L. DeLano, The PyMOL Molecular Graphics System, 0.83 ed., DeLano Scientific, San Carlos, CA, , 2002.
[38] M.S. Lalonde, Y. Zuo, J. Zhang, X. Gong, S. Wu, A. Malhotra, Z. Li, Exoribonuclease R in *Mycoplasma genitalium* can carry out both RNA processing and degradative functions and is sensitive to RNA ribose methylation, RNA 13 (2007) 1957–1968.
[39] S.F. Newbury, N.H. Smith, C.F. Higgins, Differential mRNA stability controls relative gene expression within a polycistronic operon, Cell 51 (1987) 1131–1143.
[40] N. Awano, V. Rajagopal, M. Arbing, S. Patel, J. Hunt, M. Inouye, S. Phadtare, *Escherichia coli* RNase R has dual activities, helicase and RNase, J. Bacteriol. 192 (2010) 1344–1352.
[41] A. Holzle, S. Fischer, R. Heyer, S. Schutz, M. Zacharias, P. Walther, T. Allers, A. Marchfelder, Maturation of the 5S rRNA 5' end is catalyzed in vitro by the endonuclease tRNase Z in the archaeon *H. volcanii*, RNA 14 (2008) 928–937.
[42] K. Schierling, S. Rosch, R. Rupprecht, S. Schiffer, A. Marchfelder, tRNA 3' end maturation in archaea has eukaryotic features: the RNase Z from *Haloferax volcanii*, J. Mol. Biol. 316 (2002) 895–902.