Insights into the Mechanism of Progressive RNA Degradation by the Archaeal Exosome*

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Initially identified in yeast, the exosome has emerged as a central component of the RNA maturation and degradation machinery both in Archaea and eukaryotes. Here we describe a series of high-resolution structures of the RNase PH ring from the Pyrococcus abyssi exosome, one of them containing three 10-mer RNA strands within the exosome catalytic chamber, and report additional nucleotide interactions involving positions N5 and N7. Residues from all three Rrp41-Rrp42 heterodimers interact with a single RNA molecule, providing evidence for the functional relevance of exosome ring-like assembly in RNA processing. Furthermore, an ADP-bound structure showed a rearrangement of nucleotide interactions at site N1, suggesting a rationale for the elimination of nucleoside diphosphate after catalysis. In combination with RNA degradation assays performed with mutants of key amino acid residues, the structural data presented here provide support for a model of exosome-mediated RNA degradation that integrates the events involving catalytic cleavage, product elimination, and RNA translocation. Finally, comparisons between the archaeal and human exosome structures provide a possible explanation for the eukaryotic exosome inability to catalyze phosphate-dependent RNA degradation.

Initially described as a multisubunit RNase complex required for maturation of 5.8 S rRNA in yeast (1), the 3′ → 5′ exoribonuclease complex exosome was subsequently shown to play a central role on numerous pathways related to RNA processing and degradation, both in the nucleus and in the cytoplasm (reviewed in Ref. 2). These include 3′ end processing of rRNAs, small nuclear RNAs, and small nuclearolar RNAs (3−5), degradation of aberrant pre-mRNAs, pre-tRNAs, and pre-rRNAs (6−9), normal turnover of cytosolic mRNAs (10), and degradation of RNA fragments produced during RNA interference processes (11, 12).

Increasing structural and genetic studies with archaeal and eukaryotic exosomes have unveiled the evolutionary conservation of its molecular architecture (1, 13−18). The archaeal exosome consists of two RNase PH subunits (Rrp41 and Rrp42) and two proteins containing the S1/KH (Rrp4) or S1/zinc ribbon (Csl4) RNA-binding domains (17−19). Determination of the three-dimensional structure of the Sulfolobus solfataricus and Archaeoglobus fulgidus exosomes revealed a PNPase-like fold (20), composed by alternating RNAase PH subunits assembles into a hexameric ring capped by a trimer of RNA-binding proteins, which can be formed by either Rrp4 or Csl4 or possibly by a mixture of both (19, 21, 22). Such an architecture encloses the exoribonucleolytic active sites at the bottom of the RNase PH ring catalytic chamber and restricts the entry to only unstructured RNA through the S1 pore, formed by the RNA-binding subunits of the exosome cap placed at the top of the ring (19, 21−23). Both Rrp41 and Rrp42 subunits possess the same RNAase PH-fold and are involved in substrate binding, but the amino acid residues contributing to the interactions with the phosphate nucleophile at the 3′ end of the RNA are located exclusively within the Rrp41 subunit (21).

Compared with archaeal exosomes, the eukaryotic exosome displays a more diverse subunit composition: the PNPase-like core is composed of six different RNase PH subunits regarded as Rrp41-like (Rrp41, Rrp46, and Mtr3) or Rrp42-like (Rrp42, Rrp43, and Rrp45) according to sequence homology, and three putative RNA-binding proteins (Rrp4, Rrp40, and Csl4) (21). Differently from its archaeal counterpart, the yeast exosome presents two additional hydrolytic exoribonucleases, Rrp44 (also known as Dis3) and Rrp6, which share similarities with the bacterial RNase II and RNase D, respectively (1, 24, 25). The structure of the human exosome PNPase-like core reported recently revealed an architecture very similar to the archaeal complex (26). Although phosphorolytic activity has been observed for some isolated subunits of the eukaryotic exosome (1, 27) and for the human heterodimer Rrp41/Rrp45 (26), recent studies have shown that only the hydrolytic exoribonucleases are active in the context of the eukaryotic complex (28, 29). Although this loss of phosphorolytic activity may be related to a reduction in the selection pressure compensated by the presence of additional hydrolytic subunits (30), the eukaryotic exosome PNPase-core may on the other hand have assumed a role as scaffold for interaction with the proteins assisting its various activities (28). Independently of the evolutionary and functional reasons for the conservation of the
eukaryotic exosome PNPase-like core, its lack of phosphate-dependent activity was not understood at the molecular level so far, especially taking into account that at least one eukaryotic Rrp41/Rrp42-like heterodimer (human Rrp41/Rrp45) possesses an intact archaeal-like phosphorylcorbic active site (21).

Despite the valuable information already available on the archaeal exosome, some key questions on the catalytic mechanism remain to be addressed, particularly regarding how the RNA is progressively processed, how the free nucleoside diphosphate is released, and concerning the relevance of the archaeal exosome quaternary organization. In this work, we report four x-ray structures of *Pyrococcus abyssi* Rrp41-Rrp42 complexes: two “native” structures at 2.14- and 2.40-Å resolution, which revealed nonspecific nucleotides bound to the RNA cleft; a “native” structure at 1.94-Å resolution and a CDP complex at 2.30-Å resolution. Together with RNA degradation assays, these structures give new insights into the archaeal exosome RNA processing mechanism. Furthermore, structural comparisons with the human exosome provided a possible explanation for the absence of phosphorylcorbic activity by the eukaryotic complex.

**EXPERIMENTAL PROCEDURES**

*P. abyssi* Rrp41-Rrp42 Complex Production and Purification—*Escherichia coli* vectors pET29-Rrp4, pET29-Rrp41, and pAE-Rrp42 expressing the *P. abyssi* Rrp4 and the exosome RNase PH core proteins Rrp41 and Rrp42, respectively, were constructed in previous work. Histidine-tagged Rrp41 was co-expressed with Rrp42 in *E. coli* and purified as described for homologues from *P. horikoshii* (18). Briefly, after bacterial cell disruption by sonication, the lysate was heated at 80 °C for 30 min followed by centrifugation at 20,000 × g for 30 min. The Rrp41-Rrp42 complex was purified by affinity chromatography, via binding of the histidine-tagged Rrp41 to nickel-nitrilotriacetic acid (Qiagen), followed by Superdex 75 (GE Healthcare) size exclusion chromatography. Fractions from the size exclusion chromatography presenting a hydrodynamic radius compatible with the hexameric RNase PH ring, as determined by dynamic light scattering, were pooled, concentrated, and used in crystallization trials (supplemental Fig. S1). For assembly of the nine subunits complex, Rrp4 was expressed in *E. coli* and purified as described for homologues of *P. abyssi* structure as the starting model. After an initial cycle of rigid body refinement, the structures were subjected to restrained refinement performed with REFMAC (36) applying TLS group restraints (37) for each subunit within the asymmetric unit. During the refinement procedure, strong extra densities were observed in the Fourier electron difference map in regions compatible with nucleotide binding sites. The identities and occupancy of the modeled ligands were determined based on the analysis of B-factors and the resulting electron density maps. The structures determined from crystals soaked into 10-mer poly(A) RNA and CDP were refined using the native *P. abyssi* structure as the starting model. After an initial cycle of rigid body refinement, the structures were subjected to restrained refinement performed with REFMAC, applying TLS group restraints, as described for the first structure. The ligand molecules (UDP/GMP and ADP in the native structures and CDP and 10-mer poly(A) RNA in the ligand soaked structures) were modeled into the electron density maps using COOT. For all structures, water molecules were automatically introduced by the program wARP (38). Details of the refinement statistics are shown in Table 1.

**Mutagenesis and Activity Assays**—Four point mutations (R137A, D180A, D186A, and D204A) and a double mutation (R89E/K91E) were introduced in Rrp41 and a point mutation (R137A, D180A, D186A, and D204A) and a double mutation (R89E/K91E) were introduced in Rrp42 using standard site-directed mutagenesis protocols. The reconstituted mutant exosome variants were purified following the same protocol established for the wild-type form. RNA degradation assays were performed at 60 °C in reaction buffer (10 mM Tris-Cl, pH 8.0, 10 mM NaPO₄, 50 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.8 units of RNasin, and 100 μg/ml bovine serum albumin), containing 10 nmol 32P-5'-labeled 15-mer poly(rA) as substrate. Initially, different complex concentrations (10 nm to 1 μM) were incubated in the reaction mixture for 20 min to access complex activity. The time course assays presented in Figs. 2, b and c, and 5c were performed

**Structure Determination and Refinement**—The structure of the *P. abyssi* exosome RNase PH ring was solved by molecular replacement with the program MOLREP (32) of the CCP4 suite (33). Atomic coordinates of the *A. fulgidus* Rrp41-Rrp42 heterodimer (PDB entry 2BA0) were used as the search model. *A. fulgidus* Rrp41 and Rrp42 share sequence identities of 55.4 and 49.8% with *P. abyssi* Rrp41 and Rrp42, respectively. Molecular replacement solution was subjected to iterative rounds of manual rebuilding into the 2[F – F] and [F – F] electron density maps using COOT (34) and refinement was carried out using simulated annealing, conjugate gradient minimization and temperature factor protocols available in CNS (35). Final rounds of refinement were performed with REFMAC (36) applying TLS group restraints (37) for each subunit within the asymmetric unit. During the refinement procedure, strong extra densities were observed in the Fourier electron difference map in regions compatible with nucleotide binding sites. The identities and occupancy of the modeled ligands were determined based on the analysis of B-factors and the resulting electron density maps. The structures determined from crystals soaked into 10-mer poly(A) RNA and CDP were refined using the native *P. abyssi* structure as the starting model. After an initial cycle of rigid body refinement, the structures were subjected to restrained refinement performed with REFMAC, applying TLS group restraints, as described for the first structure. The ligand molecules (UDP/GMP and ADP in the native structures and CDP and 10-mer poly(A) RNA in the ligand soaked structures) were modeled into the electron density maps using COOT. For all structures, water molecules were automatically introduced by the program wARP (38). Details of the refinement statistics are shown in Table 1.

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The abbreviation used is: BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol.

4 C. R. Ramos and C. C. Oliveira, unpublished data.


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**TABLE 1**

| Data collection and refinement statistics | UDP/GMP (native) | ADP (native) | 10-mer poly(rA) (soaking) | CDP (soaking) |
|------------------------------------------|------------------|--------------|--------------------------|---------------|
| Space group                              | P321             | P321         | P321                      | P321          |
| Cell dimensions                          |                  |              |                          |               |
| a, b, c (Å)                              | 94.9, 94.9, 127.6 | 94.2, 94.2, 127.7 | 94.8, 94.8, 129.6 | 94.5, 94.5, 127.5 |
| α, β, γ (°)                              | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |
| Resolution (Å)                           | 2.14 (2.26-2.14) | 2.30 (2.44-2.30) | 1.94 (2.06-1.94) | 2.40 (2.54-2.40) |
| R<sub>free</sub> (%)                     | 17.2 (63.9)      | 9.3 (45.1)   | 10.9 (62.7)             | 12.4 (60.7)   |
| I/O<sub>θ</sub> (%)                      | 11.3 (3.3)       | 16.5 (3.7)   | 14.9 (3.5)             | 11.1 (2.8)    |
| Completeness (%)                         | 99.3 (96.0)      | 97.5 (89.6)  | 98.2 (90.1)           | 99.2 (96.0)   |
| Redundancy                               | 14.8 (11.8)      | 9.7 (8.4)    | 11.0 (9.2)           | 5.5 (5.2)     |

| Refinement | Resolution (Å) | 19.87-2.14 | 19.98-2.30 | 20.00-1.94 | 19.80-2.40 |
|------------|----------------|------------|------------|------------|------------|
| No. reflections | 33,921 | 26,541 | 44,547 | 23,727 |
| R<sub>free</sub>/R<sub>free</sub> | 0.188/0.255 | 0.190/0.242 | 0.187/0.242 | 0.182/0.258 |
| Number of atoms | 3913 | 3900 | 3915 | 3903 |
| Protein | 89 | 78 | 348 | 65 |
| Water | 239 | 151 | 301 | 176 |
| B-factors (Å<sup>2</sup>) | 35.6 | 42.4 | 30.9 | 40.6 |
| RNA/nucleotides | 49.9 | 42.2 | 84.1 | 32.3 |
| MPD | 55.6 | 55.1 | 55.9 | 58.3 |
| Water | 36.9 | 37.9 | 37.1 | 36.7 |
| R.m.s. deviations | 0.023 | 0.021 | 0.020 | 0.021 |
| Bond lengths (Å) | 2.312 | 2.156 | 2.065 | 2.106 |
| Ramachandran (%)<sup>a</sup> | 90.4 | 92.2 | 91.7 | 89.5 |
| Most favored regions | 9.4 | 7.4 | 7.6 | 10.1 |
| Additional allowed regions | 0.0 | 0.2 | 0.5 | 0.2 |
| Generously allowed regions | 0.2 | 0.2 | 0.2 | 0.2 |

<sup>a</sup> Values in parentheses are for the highest resolution shell.
<sup>b</sup> Calculate with PROCHECK (45).

with complex concentrations of 100 nM. The reactions were resolved on 8% denaturing TBE-Urea polyacrylamide gels.

Accession Codes—Protein data bank coordinates and structure factors have been deposited with accession codes 2PNZ, 2POO, 2PO1, and 2PO2 for the *P. abyssi* exosome RNase PH ring bound to UDP/GMP, ADP, 10-mer poly(rA), and CDP, respectively.

**RESULTS**

**P. abyssi** Rrp41-Rrp42 Complex Overall Structural Analyses

The subunits Rrp41 and Rrp42 of the *P. abyssi* exosome were co-expressed in *E. coli* and the purified complex was crystallized in the trigonal *P*321 space group. The structure was solved by molecular replacement using the atomic coordinates of the *A. fulgidus* Rrp41-Rrp42 heterodimer as the search model (PDB code 2BAO). The first native structure was refined at 2.14-Å resolution to an *R*<sub>free</sub> of 25.5%, with good stereochemistry. The asymmetric unit contains one Rrp41-Rrp42 heterodimer and application of the crystallographic 3-fold axis reconstitutes the characteristic exosome RNase PH core, which is composed of a hexameric ring of alternating subunits (21) (supplemental Fig. S2). The overall structure of the *P. abyssi* exosome RNase PH ring is very similar to its archaeal counterparts from *A. fulgidus* and *S. solfataricus* (19, 21). Superposition of individual Rrp41 and Rrp42 subunits results in root mean square deviations ranging from 0.899 to 1.619 Å for the superimposed Ca atoms (supplemental Fig. S3 and supplemental Table S1). The high quality of the electron density maps allowed modeling of residues 9 to 244 from Rrp41 and residues 8 to 274 from Rrp42, without gaps in the polypeptide chains. During the refinement procedure, strong extra electron densities in the Fourier difference map |<i>F</i>|<sub>c</sub> - |<i>F</i>|<sub>e</sub> revealed the presence of nucleotides at the phosphorolytic active site and at an additional interaction site (Fig. 1a). Interestingly, structural comparison with the RNA-bound structure of *S. solfataricus* RNase PH ring (PDB code 2C38) revealed that the additional nucleotide binding site identified in the *P. abyssi* complex is adjacent to the fourth nucleotide within the exosome-specific RNA recognition cleft, which is formed at the interface of the Rrp41-Rrp42 subunits (23). Based on this finding, we assigned this additional nucleotide as N5, following the nomenclature introduced by Lorentzen and Conti (23), which defines N1 as the nucleotide of the RNA 3′ end that occupies the phosphorolytic active site. Given the unspecific nature of RNA recognition by the exosome, the electron densities observed at the N1 and N5 binding sites probably arose from a mixture of nucleotides copurified with the recombinant Rrp41-Rrp42 complex. In both cases the phosphate and ribose groups were clearly defined by the electron density maps, which allowed also modeling of an UDP and a GMP in the N1 and N5 sites, respectively.

A second native *P. abyssi* RNase PH ring structure was solved and refined to a resolution of 2.30 Å and an *R*<sub>free</sub> of 24.2%. Similarly to the first structure, a strong electron density was observed close to the phosphorolytic active site during refinement, which allowed modeling of an ADP molecule (discussion in next sections). To obtain additional information on nucleoside diphosphate binding to the exosome phosphorolytic site, we also determined the structure from a crystal soaked into CDP. These crystals diffracted up to a resolution of 2.40 Å and the structure was refined to an *R*<sub>free</sub> of 25.8%. The electron...
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density maps allowed us to unequivocally model a CDP molecule at the N1 binding site, mimicking an RNA phosphorolysis product (Fig. 1b).

Finally, to better determine the RNA interactions with the exosome core and to confirm the presence of a N5 nucleotide binding site at the specific RNA recognition cleft, we solved the structure of the P. abyssi RNase PH ring in complex with a 10-mer poly(A) RNA. Binding of the 10-mer poly(A) RNA to the RNase PH ring was achieved by soaking native crystals with a 14 mM solution of the 10-mer poly(A). Because the crystals were grown in the absence of phosphate, phosphorolytic RNA cleavage was unlikely to happen. The 10-mer poly(A) RNA-soaked crystals diffracted to 1.94-Å resolution and the structure was refined to an Rfree of 23.6%. The RNA strand was found in the RNA recognition cleft, located at the interface of the Rrp41-Rrp42 heterodimer. Taking into account that the modeled nucleotides were refined with 100% occupancy and that, in the P. abyssi exosome crystals, the full structure is reconstituted by the crystallographic 3-fold axis, the RNA-bound structure indicates that the archaeal exosome RNase PH ring binds three RNA strands concomitantly. This result agrees with isothermal titration calorimetry analysis reported by Oddone and colleagues (39), which showed that the S. solfataricus RNase PH ring binds RNA with a stoichiometry of three RNA molecules to one hexameric ring.

The RNA Recognition Cleft Is Formed by Residues from All Three Heterodimers

As in the case of the S. solfataricus RNase PH ring structure in complex with RNA (23), the contacts found in the RNA recognition cleft of the P. abyssi exosome ring described here are dominated by ionic interactions between the phosphate backbone of the four nucleotides of the RNA 3′ end and a ladder of arginine side chains from both Rrp41(1) and Rrp42(1) subunits (for clarity, the three heterodimers that form the quaternary structure of the archaeal exosome core are assigned as 1, 2, or 3 throughout the text and the residues and subunits belonging to them will be distinguished by these numbers in parentheses). However, the 10-mer poly(A) RNA-bound structure solved in this work showed a fifth nucleotide (N5) at the specific RNA recognition cleft, which had already been observed as a free nucleotide in the first native structure. Furthermore, the high resolution obtained for this complex revealed a more intricate network of RNA-exosome contacts, showing that subunits from the three heterodimers participate in RNA recognition (Fig. 2, a and b and supplemental Table S2). The phosphorolytic site of the P. abyssi exosome on the Rrp41(1) subunit is formed by two conserved arginines (Arg97 and Arg137) and by the catalytic residue Asp180, which interacts with the phosphate moiety of nucleotide N1. The ribose 3′ OH of N1 is hydrogen-bonded to the main chain of the conserved Ala134 and the inorganic phosphate binding site, comprised by the conserved residues Gly135, Thr136, and Arg137, is occupied by three water molecules (Fig. 2c). These water molecules are located in equivalent positions of the phosphate PB oxygen atoms modeled in the CDP-bound structure (Fig. 1b). The interaction proposed for the ribose 2′ OH of N1 with the carbonyl group of Ala134 of S. solfataricus Rrp41 (correspondent to P. abyssi Ala132) (23), was not observed in the P. abyssi structure. Instead, all the structures solved in this work showed a water-mediated interaction between the ribose 2′ OH of N1 and the conserved lysine Lys45 from the Rrp42(3) subunit, which is part of the neighbor heterodimer (Fig. 2c). On the other extreme of the exosome-specific RNA recognition cleft, the nucleotide N5 found in the P. abyssi RNase PH core structure breaks the pattern of base stacking interaction observed for the four innermost 3′ end nucleotides, pointing the RNA strand to the exosome central chamber and, most importantly, the phosphate moiety of N5 interacts with conserved residues Arg89 and Lys91 of the Rrp41(2) subunit from the adjacent heterodimer (Fig. 2a).

To verify the relevance of the Rrp42 Lys45 and Rrp41 Arg89 and Lys91 residues to catalysis, RNA degradation assays were carried out with reconstituted Rrp41-Rrp42K89E-K91E and Rrp41R89E-K91E Rrp42 mutant complexes. These amino acid substitutions, as well as other mutations performed in this work, did not affect the RNase PH ring assembly and stability because the reconstituted exosome variants were purified following the same protocols used for the wild-type complex and
displayed similar size exclusion chromatography patterns and hydrodynamic radii. The substitution of residues Arg89 and Lys91 from Rrp41 by glutamic acids blocked RNA degradation completely, similarly to mutation of catalytic residue Asp180 in the Rrp41D180A-Rrp42 complex (Fig. 3a, lanes 14–17 compared with lanes 6–9), whereas the exosome variant bearing point mutation Rrp42K45A displayed a slower catalytic rate (Fig. 3, a, lanes 10–13 compared with lanes 2–5, and b, lanes 10–18 compared with lanes 1–9). Degradation assays with the nine-protein complex (Rrp41-Rrp42-Rrp4) presented results similar to the hexameric ring (Fig. 3c, lanes 1–18). Thus, addition-ally to the central heterodimer Rrp41(1)-Rrp42(1), conserved archaeal residues from both flanking heterodimers (Lys45 from subunit Rrp42(3) and Arg89/Lys91 from subunit Rrp41(2)) (Fig. 2b and supplemental Fig. S4) are involved in formation of the specific RNA recognition cleft and are essential for efficient RNA degradation. These findings provide the first direct evidence of the role of archaeal exosome quaternary structure in RNA processing.

**A Model for RNA Substrate Interaction with the Archaeal Exosome Catalytic Chamber**

Besides nucleotides N1–N5 found at the *P. abyssi* exosome RNA recognition cleft, the electron density maps of the RNA-bound structure allowed modeling of two additional nucleotides, which were unambiguously assigned to positions N7 and N10 (Fig. 4a). Following the path from the exosome phosphorolytic site toward the top entry of the RNase PH ring channel, nucleotides N7 and N10 were, respectively, found in a midway channel groove and in the neck structure formed at the top of the ring (Fig. 4b). Whereas the groove at N7 is formed by conserved loops from the Rrp42(1) (residues Pro96–Gly97–Pro98–Pro99) and Rrp41(2) (residues Pro92–Gly93–Pro94) subunits at the interface of two heterodimers, the neck at N10 is formed in the interface of the three adjacent Rrp41 subunits by the loops containing residues His63–Pro64–Lys65–His66. This finding reinforces the requirement of a quaternary structure for exosome function on RNA degradation, because residues from neighboring heterodimers participate in nucleotide binding. Interestingly, nucleotides N7 and N10 interact with the exosome mostly via their purine moieties, in contrast with the salt bridge interactions observed between the N1–N5 phosphate backbone and the exosome RNA recognition cleft. Nonetheless, the unspecific nature of the interaction is preserved, given that N10 and N7 binding are mediated by hydrophobic and π-stacking interactions that are not specific to a particular base.

Based on the position of nucleotides N1–N5, N7, and N10, it was possible to model the entire 10-mer poly(A) RNA strand within the exosome catalytic chamber (Fig. 4b). Despite the central channel constriction, especially at the N7 groove and the N10 neck, the model shows that the catalytic chamber of the archaeal exosome could accommodate three RNA strands (Fig. 4c). Moreover, the connectivity of the modeled RNA strands represents the most probable configuration that avoids steric clashes (see supplemental Fig. S5 for a detailed description). According to this model, nucleotides N6, N8, and N9 do not appear to interact directly with the exosome. Instead, they occupy broad cavities (Fig. 4c), which may confer to them a certain mobility, explaining the lack of electron density in the
Interestingly, Oddone and co-workers (39) showed that whereas the archaeal RNase PH ring binds three RNA strands, its association with the Rrp4 subunits reduces the RNA:exosome stoichiometry to 1:1. Thus, the RNase PH ring is not the structural constraint that limits entry to only one RNA strand at a time in the exosome catalytic chamber. Furthermore, the unusual mode of interaction between the three RNA strands could explain the dramatic reduction of RNA affinity by the PH ring compared with the S1-containing complex (39). In conclusion, the results presented in this work showed that the 3’ end of the RNA strand enters the exosome catalytic chamber through the top side of the RNase PH ring, which fits three 10-mer poly(rN) oligoribonucleotides, in agreement with previous evidence obtained from RNase protection assays (23) and from recent structural data on the S. solfataricus exosome complexed with a structured RNA containing a 3’ poly(rA) extension (22).

**RNA Processing Mechanism**

The Role of Exosome N7 and N10 Binding Sites—Previous studies (19, 22) have shown the relevance of the archaeal RNase PH ring neck in directing the RNA substrate to the exosome catalytic chamber. Now, the identification of a new and conserved nucleotide binding pocket (N7 groove) within the exosome central channel raises a question about the role of N10 and N7 sites in the RNA processing mechanism. Comparison between the native and RNA-bound structures revealed a significant decrease in the B-factor values of the loops forming the N7 groove and N10 neck structures (Fig. 5). Relative to the native exosome structures, the B-factor values of the RNA-bound structure dropped by ~14 (±5) and 2 (±1)% for the N7 and N10 nucleotide binding sites, respectively (B-factor values were normalized to the mean overall value for each structure to adjust for differences in resolution). Furthermore, the constricted N10 neck undergoes a structural rearrangement to accommodate the RNA strands, which takes place mainly through conformational changes in the side chains of His63 and His66 of the Rrp41 subunit (Fig. 5). Interestingly, in some archaeal exosome structures reported previously (PDB codes 2BA0 and 2BR2), the Rrp42 loop that forms the N7 groove could not be modeled due to lack of electron density. These observations, together with the data presented in this work indicate that the N7 and N10 nucleotide binding sites found in the central channel of the archaeal exosome are flexible structures that undergo structural stabilization upon RNA binding.

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![RNA degradation activity of P. abyssi wild-type and mutant exosome complexes](image)
FIGURE 4. 10-mer poly(A) RNA bound to the \textit{P. abyssi} exosome catalytic chamber. \textit{a}, $|F_o| - |F_c|$ electron density map contoured at 2.5 $\sigma$ superposed on the final 10-mer RNA model. Modeling of N7 and N10 bases is highlighted. \textit{b}, side cut of the exosome core structure showing the accommodation of the 10-mer poly(A) RNA. N7 and N10 binding sites are highlighted. Rrp41 and Rrp42 subunits are, respectively, colored in blue and light brown and subunits from the same heterodimer are labeled with the same number (1 or 2). \textit{c}, top view of the RNase PH ring structure showing the binding of nucleotides N1 to N10 through successive cuts. The three RNA strands are showed in sticks and colored yellow, green, and blue. Subunits from the same heterodimer are labeled with the same number (1 to 3).
less efficient than the wild-type complex (Fig. 3, a, lanes 18–21 compared with lanes 2–5, and b, lanes 19–27 compared with lanes 1–9). Interestingly, the degradation pattern presented by this variant clearly shows accumulation of unexpected RNA subproducts spanning 7–10 nucleotides (Fig. 3, a, lane 21, and b, lanes 23–25), which are not detected for the wild-type exosome (Fig. 3, a, lanes 2–5 and b, lanes 1–9). After a long incubation, the subproducts were further processed down to 4–5 nucleotide fragments (Fig. 3b, lanes 26 and 27). Moreover, although the final degradation products (4–5 nucleotides) correspond to the minimal length necessary to occupy the specific RNA recognition cleft, the subproducts generated by the Rrp41R137A-Rrp42 variant are compatible with oligoribonucleotides spanning from the N1 to the N7–N10 sites. The structural and biochemical data suggest a model for exosome-mediated RNA degradation in which the N7 groove and N10 neck might be working synergistically with the recognition cleft to stabilize the RNA within the exosome catalytic chamber in a manner that promotes progressive RNA degradation. By destabilizing the N1 binding site and consequently disrupting this synergy, RNA degradation slows down and turns from a continuous to a stepwise process for production of the final 4–5 oligoribonucleotides. To verify if the same effect is observed in the context of the full exosome, we performed the same experiments with the Rrp4-containing Rrp41R137A-Rrp42 variant. Similarly to the degradation pattern observed for the RNase PH ring, the Rrp41 R137A mutation led to formation of intermediate products (Fig. 3c, lanes 19–27). These results suggest that the model proposed for RNA binding and processing involving N7 and N10 binding sites may be extrapolated to the biologically active nine-subunit complex.

A Rationale for Nucleoside Diphosphate Elimination after Catalysis

The CDP-bound structure solved in this work suggests that, following phosphorolytic cleavage, the newly formed nucleoside diphosphate undergoes stabilization within the exosome active site prior to release. In comparison with the N1 nucleotide modeled in the 10-mer poly(A) RNA-bound structure, CDP displays similar structural conformation, but presents a more extensive network of interactions with the exosome (Fig. 1b) and lower normalized B-factor. This apparent structural stabilization of the catalysis product is inconsistent with the elimination of the 3’ end RNA-cleaved nucleotide by simple diffusion, especially if one takes into account the high processive capacity of archaeal exosomes toward RNA degradation. Unexpectedly, the electron density maps of the second native P. abyssi exosome structure solved in this work showed clearly a nucleoside diphosphate bound to the N1 site (modeled as an ADP) with two possible conformations regarding the phosphates (Fig. 6a), suggesting a nucleoside diphosphate structural rearrangement prior to diffusion. In Fig. 6a, position A corresponds to the expected conformation of the cleavage product after phosphorolytic attack, as shown by Lorentzen and Conti (23) and also observed in our CDP-bound structure. The ADP alternative conformation places the phosphate PB moiety in position to interact with three conserved acidic residues from the Rrp4 subunit: Asp186, Asp204, and catalytic Asp180 (position B in Fig. 6a). Although phosphate binding sites are not expected to be composed of acidic residues (40), the quality of the electron density maps clearly indicated the conformational change of phosphate PB. Such a structural rearrangement could take place by a simple rotation along the PA–O4 bond, which is
compatible with previous findings describing a displacement of about 4 Å of the newly formed 3’ end of the RNA molecule (N2) after phosphorolytic cleavage (23). Furthermore, this alternative conformation points the nucleotide toward a “side channel,” which was postulated as a possible diffusion route for the excised product (23). Interestingly, primary structure alignment revealed that in Archaea, conservation of the RNase PH ring residues that form this channel is comparable with sequence conservation within the regions involved in the interaction with RNA-binding proteins Rrp4 and Csl4 (Fig. 6b), suggesting that the side channel may indeed have a functional role. To verify the effect of Rrp41 D186A and D204A mutations on the catalytic activity, RNA degradation assays were carried out with the reconstituted Rrp41D186A-Rrp42 and Rrp41D204A-Rrp42.
Rrp42 complexes. Substitution of residue Asp\textsuperscript{186} in Rrp41 abolishes completely RNA degradation similar to the results observed for the mutant Rrp41\textsuperscript{180A}, Rrp42, whereas the variant Rrp41\textsuperscript{180A}, Rrp42 displayed a slower activity (Fig. 6c).

These new structural and functional data allow the proposition of a rationale for the release of the excised nucleoside diphosphate, connecting the exosome mechanisms of RNA catalytic cleavage to progressive processing. It was previously proposed that the phosphorolytic RNA attack proceeds via a S\textsubscript{2} mechanism, with a conserved acidic residue performing the role of the general proton donor to the 3’ oxygen of the cleaved RNA N2 ribose (23). Following catalysis, several events must occur to promote continuous RNA processing, including elimination of the nucleoside diphosphate product, re-protonation of the catalytic acidic amino acid residue, re-positioning of the inorganic phosphate at the phosphorolytic site, and RNA translocation to the N1 site. The structural rearrangement of the cleavage product at the N1 site seems to be compatible with such requirements. First, given the negative charge of phosphate PB moiety, interaction with the new site (position B in Fig. 6a) would require protonation of the aspartic acid residues, including the catalytic residue Asp\textsuperscript{180} of Rrp41. Additionally, the conformational change of the nucleoside diphosphate creates space for binding of an incoming inorganic phosphate. These observations suggest that after catalysis, the product undergoes a structural rearrangement induced by protonation of residues Asp\textsuperscript{204}, Asp\textsuperscript{186}, and catalytic Asp\textsuperscript{180} of Rrp41. The pK\textsubscript{a} value for the conserved Asp\textsuperscript{186} residue calculated according to Li et al. (41) is ~11 and is in agreement with the pK\textsubscript{a} observed for the equivalent residue in \textit{S. solfataricus} exosome (23). This pK\textsubscript{a} value, unusual for an aspartate, together with the close proximity of Asp\textsuperscript{186} to Asp\textsuperscript{180} and Asp\textsuperscript{204}, suggests that this residue may serve as a proton donor to the aspartic acids involved in the alternative PB site. RNA degradation assays corroborate this model, because the inactivity of the variant Rrp41\textsuperscript{D186A}–Rrp42 toward the RNA substrate indicates that residue Rrp41 Asp\textsuperscript{186} directly participates in catalysis. Furthermore, the low activity showed by the mutant Rrp41\textsuperscript{D204A}–Rrp42 is in agreement with the role of the Asp\textsuperscript{204} side chain in stabilizing the nucleoside diphosphate PB moiety in its new conformation, prior to product elimination (Fig. 6c). The nucleoside diphosphate conformational change would release the product from the base-stacked position assisted by binding of a new inorganic phosphate at its specific site. Thus, the newly formed RNA 3’ end, which has been shown to remain bound to the exosome after N1 cleavage (23), may move to the exosome catalytic site already prepared for the next round of 3’ end RNA cleavage (Fig. 7).

**Molecular Basis for the Phosphate-dependent Inactivity of the Eukaryotic Exosome**

Structural comparison between the \textit{P. abyssi} RNA-bound RNase PH ring and the human exosome (PDB code 2NN6) showed that the N7 nucleotide binding site identified in the \textit{P. abyssi} structure is conserved only at the interface of the human Rrp43-Mtr3 heterodimer (Fig. 8a). Although many eukaryotic RNase PH subunits have diverged from their archaeal counterparts, the human Rrp41–Rrp45 heterodimer possesses an intact RNA recognition cleft (21), which would be consistent with the presence of only one N7 groove. However, the location of the conserved human N7 groove relative to the Rrp41–Rrp45 RNA clef is not compatible with the RNA binding model described for the archaeal exosome, being too far from the N5 binding site (Fig. 8b). Furthermore, the neighbor Rrp41-like subunit (Rrp46) presents a deletion in a loop at the correspondent position of the archaeal positively charged residues (\textit{P. abyssi} Arg\textsuperscript{97} and Lys\textsuperscript{91}) that participate in the N5 nucleotide binding site (Fig. 8c). In addition to the lack of conservation of the N5 and N7 binding sites, the second acidic residue (Rrp41 Asp\textsuperscript{186}) essential for the archaeal catalytic mechanism (Figs. 6c and 7) is also not conserved in the human Rrp41 subunit (Fig. 8e). Taken together, these analyses provide an explanation for the absence of phosphorolytic activity shown by the eukaryotic exosome and suggest that the RNA substrate might not bind within its PNPase-core central channel.

**DISCUSSION**

The crystal structures of the \textit{P. abyssi} Rrp41–Rrp42 RNase PH ring solved in this work, together with RNA degradation assays, contribute significantly to the understanding of the
RNA processing mechanism by the archaeal exosome. The structural analyses showed that residues from all three Rrp41-Rrp42 heterodimers participate in the binding of the same RNA molecule, revealing a functional role for the exosome quaternary organization. Moreover, identification of the N7 and N10 nucleotide binding sites and degradation assays performed with the Rrp41\textsuperscript{R137A}-Rrp42 complex indicated that the exosome central channel, besides trapping and directing the RNA substrate to the phosphorolytic site, appears to act synergistically with the N1 binding site to promote RNA stabilization and processing. In addition, alternative conformations observed for nucleoside diphosphate bound to the N1 site together with degradation assays performed with the mutants Rrp41\textsuperscript{D186A}-Rrp42 and Rrp4-Rrp41\textsuperscript{D204A}-Rrp42 indicated that a structural rearrangement after catalysis precedes product release. These results revealed also that residue Asp\textsuperscript{186} from Rrp41 directly participates in RNA catalytic cleavage. Based on this and on previous studies (19, 21–23, 39), we propose a model for archaeal exosome-mediated RNA processing. Initially, single-stranded RNA binds to the S1 pore formed by the RNA-binding subunits (Rrp4, Csl4, and Rrp40) and is threaded into the exosome catalytic chamber through the N10 neck located at the top of the RNase PH ring. In this cavity, the RNA is stabilized mainly by electrostatic interactions between the five 3’ end nucleotides and the RNA recognition cleft and by anchoring of the seventh and 10th nucleotides to the conserved binding pockets at the exosome central channel. After phosphorolytic cleavage, nucleoside diphosphate undergoes a structural rearrangement within the N1 site and is released through a conserved side channel in concert with the entry of a new inorganic phosphate. As the product is released, the RNA, which loses interactions but remains within the chamber, is repositioned for a new round of catalytic cleavage. In a way similar to that described for the bacterial RNase II (42), the final 4–5 nucleotide products generated by the exosome indicate that, when the RNA becomes shorter than the minimal length necessary for interaction at the RNA recognition cleft, translocation is no longer possible. Although archaeal exosomes possess three equivalent phosphorolytic sites, it is still not clear if all of them have a role in the cleavage of a single RNA substrate, and further experiments should be performed to address this question.

The new features of the archaeal phosphorolytic RNA degradation mechanism identified in this work allowed us to propose an explanation for the lack of such activity in the eukaryotic complexes. Additionally, it has been speculated that the RNA substrate could reach the eukaryotic active subunits, especially the yeast Rrp44, either through the exosome PNPase-core central channel or by direct access (43). The lack of conservation of some nucleotide binding sites within the eukaryotic PNPase-core may indicate that this route is not used by the RNA substrates, suggesting that the complex has exclusively assumed the function of scaffold for the exosome activating complexes. Structure determination of hydrolytic subunit-containing eukaryotic exosomes will help to confirm or refute this hypothesis.
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