CHAPTER 3

THE ARCHAEAL EXOSOME

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Abstract: The archaeal exosome is a protein complex with structural similarities to the eukaryotic exosome and bacterial PNPase. Its catalytic core is formed by alternating Rrp41 and Rrp42 polypeptides, arranged in a hexameric ring. A flexible RNA binding cap composed of the evolutionarily conserved proteins Rrp4 and/or Csl4 is bound at the top of the ring and seems to be involved in recruitment of specific substrates and their unwinding. Additionally, the protein complex contains an archaea-specific subunit annotated as DnaG, the function of which is still unknown. The archaeal exosome degrades RNA phosphorolytically in 3′ to 5′ direction. In a reverse reaction, it synthesizes heteropolymeric RNA tails using nucleoside diphosphates. The functional similarity between the archaeal exosome and PNPase shows that important processes of RNA degradation and posttranscriptional modification in Archaea are similar to the processes in Bacteria and organelles.

INTRODUCTION

The Archaea are a unique group of prokaryotic micro-organisms also named "the third domain of life", since they have molecular characteristics that distinguish them from the Bacteria as well as from the Eukarya.[1-3] The best known Archaea are the methanogens and the extremophiles (hyperthermophies, halophiles, acidophilies), but it should be noticed that the vast majority of archaeal species live as ubiquitous mesophiles in water and soil.[3] Archaea are phylogenetically more closely related to Eukarya than to Bacteria,[2] and many of the archaeal proteins and protein complexes are simplified versions of their eukaryotic counterparts.[4,5] Since recombinant protein and protein complexes of hyperthermophilic Archaea can be relatively easily overproduced and crystallized, they are important research objects.

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Proteins involved in RNA metabolism belong to the evolutionarily most conserved ones and orthologs of several ribonucleases are found in all three domains of life.[6] Koonin at al. proposed the existence of an archaeal exosome in 2001, based on the finding that in most of the sequenced archaeal genomes, three orthologs of eukaryotic exosomal subunits (Rrp4, Rrp41 and Rrp42) are encoded in an array in a highly conserved superoperon.[7] A fourth ortholog of a eukaryotic exosome subunit, Csl4, was found to be encoded in another operon in these genomes. The first experimental evidence for the existence of an exosome-like complex in Archaea was presented in 2003,[8] and in the following years the archaeal exosome was characterized as a phosphorolytic 3′ to 5′ exoribonuclease, which can in an opposite reaction synthesize RNA[9-14] similarly to bacterial polynucleotide phosphorylase (PNPase).[15,16] Here present knowledge of the archaeal exosome is summarized.

CORE SUBUNITS OF THE ARCHAEAL EXOSOME

So far, the in vivo composition of the archaeal exosome was examined for the thermophilic species *Sulfolobus solfataricus*[8,12] and *Methanothermobacter thermoautotrophicus*.[17] The *S. solfataricus* exosome was purified by immunoprecipitation using polyclonal antibodies against the archaeal Rrp41 subunit and coprecipitating proteins were identified by mass spectrometry. The following proteins were found to copurify with Rrp41: Rrp42, Rrp4, Csl4, the archaeal DnaG-like protein, Cpn and a Cdc48 homolog.[8] The exosome of *M. thermoautotrophicus* was separated by Blue Native/SDS-PAGE and mass spectrometry analysis revealed the presence of Rrp41, Rp42, Rrp4, DnaG and the archaeal splicing endonuclease in the complex.[17] In some experiments *S. solfataricus* Csl4 is copurified in very low amounts with the exosome,[12] and this may explain the failure to detect Csl4 in the exosome of *M. thermoautotrophicus*.

It is accepted that Rrp41, Rrp42, Rrp4 and Csl4 belong to the core of the archaeal exosome, since they can be assembled into a complex structurally similar to the eukaryotic nine-subunit exosome.[10,18,19] The archaeal nine-subunit exosome is also structurally and functionally similar to bacterial PNPase,[20] which degrades RNA phosphorolytically but is also responsible for RNA tailing in vivo.[15,16] The nine-subunit form of the archaeal exosome contains three Rrp41, three Rrp42 and three Rrp4 and/or Csl4 polypeptides.[10] Alternating Rrp41 and Rrp42 form the catalytically active hexameric ring,[9,10] on the top of which three Rrp4 and/or Csl4 are located, forming an RNA-binding cap with a central pore.[10,18]

The archaeal DnaG protein was consistently copurified with the exosome and cosediments with Rrp41 and Rrp4 after fractionation of *S. solfataricus* cell-free extracts in glycerol density gradients by ultracentrifugation.[21] Its binding to the exosome is very strong and comparable to the interactions between Rrp41, Rrp42, Rrp4 and Csl4—in coimmunoprecipitation experiments, all five polypeptides still build a complex after washing with 1.8 M NaCl and elute together at low pH.[21] The possibility that *S. solfataricus* DnaG accidentally sticks to the exosome was excluded: it was shown that coimmunoprecipitations with Rrp41-directed or with DnaG-directed antibodies result in purifications of very similar protein complexes and that depletion of Rrp41 from the cell extract is paralleled by DnaG-depletion.[12] Although DnaG strongly interacts with the archaeal exosome and can be considered as its tenth core subunit, the physiological role of the protein remains unknown. As previously discussed,[8,12] its domain composition suggests an RNA-helicase or endoribonuclease function.

The significance of copurification of Cpn and Cdc48 with the *S. solfataricus* exosome is not clear, since both proteins have chaperone properties.[12] Based on the cosedimentation of large amounts of the *S. solfataricus* exosome with 30S and 50S ribosomal subunits in glycerol density gradients, it was proposed that this protein complex is involved in rRNA maturation like its eukaryotic counterpart.[8] The interaction of the *M. thermautotrophicus* exosome with a homomultimer of the splicing endonuclease (bulge-helix-bulge endonuclease) in a 900 kDa complex[17] strongly suggests that the endonuclease and the exosome participate in RNA processing in a coordinate manner.

STRUCTURE AND MECHANISM

The structure and the mechanism of the archaeal exosome were investigated in vitro using protein complexes reconstituted from purified recombinant subunits. Based on early reports about recombinant subunits of the eukaryotic exosome,[22,23] it was expected that at least one of the RNase PH domain (RPD)-containing subunits of the archaeal exosome, Rrp41 or Rrp42, should exhibit phosphorolytic activity and that Rrp4 or Csl4 may show hydrolytic activity. However, none of the recombinant archaeal proteins Rrp41, Rrp42, Rrp4 or Csl4 showed any RNase activity in vitro, when used separately. It turned out that reconstitution of a protein complex containing Rrp41 and Rrp42 is needed for the phosphorolytic activity of the archaeal exosome.[9,12] The activity is modulated in the presence of the RNA-binding subunits Rrp4 and/or Csl4, which do not act as RNases.[10,12] Not only is RNA degradation by the Rrp41/Rrp42 complex more efficient in the presence of Rrp4 and/or Csl4,[10,12,13] RNA binding by Rrp4 is also strongly increased when this protein is part of the exosome,[24] demonstrating the interdependence of the exosomal subunits with respect to the function of the exosome.

In the last years, recombinant exosomes from the hyperthermophilic Archaea *S. solfataricus*, *Archaeoglobus fulgidus* and *Pyrococcus abyssi* were analysed by crystallography, small angle X-ray scattering and mutagenesis, giving important information about the structure of this protein complex and its mechanisms.[9,10,13,18,25,26]

The Catalytically Active RPD-Hexamer

Crystallographic analyses revealed that the two RPD-containing subunits of the archaeal exosome Rrp41 and Rrp42 are arranged in a hexameric ring composed of three Rrp41/Rrp42 dimers. This hexameric ring is the minimal catalytic subunit of the complex, it exhibits phosphorolytic exoribonuclease activity in 3′ to 5′ direction and it strongly resembles the structure of the hexameric ring build of the two RPD domains present in each polypeptide of the homotrimeric bacterial PNPase.[9,10,12,20] The phosphate binding sites were visualised using a phosphate-mimicking ion and structure-guided mutations localised the active sites in Rrp41, in close vicinity to Rrp42, near the bottom of the central channel of the hexamer (Fig. 1).[9]

Structures of Rrp41/Rrp42 hexamers bound to RNA were also resolved showing that the four most distal nucleotides of an RNA substrate (N1-N4, numbering form the 3′-end) are bound in a cleft of an Rrp41-Rrp42 dimer by ionic interactions with a ladder of arginine residues.[27] Arginine side chains contributed by both Rrp41 and Rrp42 are involved in these interactions, explaining the importance of complex formation for enzymatic activity. Mutations of these arginines to glutamates abolish RNA degradation. The substrate

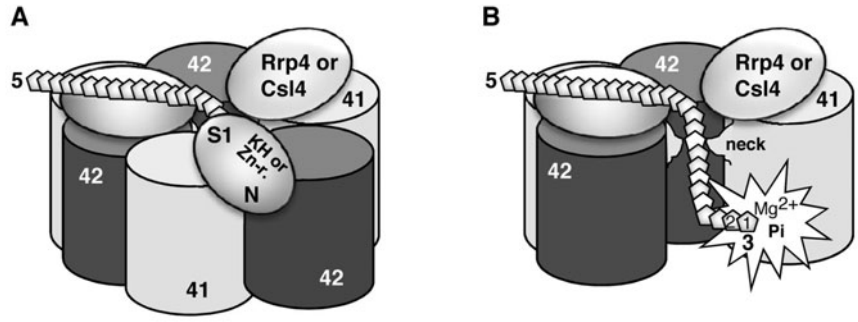

Figure 1. Schematic representation of the structure of the archaeal exosome with bound RNA. A) The nine-subunit exosome with the protruding 5′-end of an RNA substrate. Alternating RPD-containing subunits Rrp41 and Rrp42 (barrels marked with 41 and 42, respectively) are arranged in a hexameric ring. On the top of the ring, three RNA binding subunits (ovals representing Rrp4 or Csl4) are located. Rrp4 contains the N-terminal domain (N), the S1 domain and the KH domain; Csl4 contains the N-terminal domain, the S1 domain and the Zn-ribbon domain (Zn-r.). RNA (shown as a chain of pentoses) is bound to one of the Rrp4 or Csl4 subunits and its single stranded 3′-end is threaded through the S1-pore into the central channel of the hexamer. B) An Rrp41 subunit, an Rrp42 subunit and an RNA-binding subunit are removed to allow a view into the central channel of the hexamer. A narrow constriction (neck) formed by loops of the Rrp41 subunits interacts with the tenth nucleotide as numbered from the 3′-end of the substrate. These interactions as well as the interactions of the seventh and the fifth nucleotide with more than one Rrp41-Rrp42 dimer are important for RNA degradation. The active sites are located in the Rrp41 subunits. Inorganic phosphate (Pi), the first and the second nucleotide are bound directly at one of the active sites (asterisk) and the phosphoester bond between them is cleaved phosphorolytically. Most probably, the phosphate is activated by a magnesium ion also bound at the active site (for references see the text).

binding is performed mainly by electrostatic interactions with the phosphate groups of the ribose-phosphate backbone, ensuring sequence-unspecific RNA degradation. The exosome specificity towards RNA is explained by interactions with 2′-OH of the sugar.[27]

The two most distal nucleotides of the substrate (N1 and N2) are bound directly at the active site (Fig. 1) and the bond between them is cleaved phosphorolytically, releasing a ribonucleotide 5′-diphosphate (rNDP). The active site of the archaeal Rrp41 is formed by two conserved arginine residues and a catalytically active aspartate residue (D182 in *S. solfataricus* Rrp41). It was proposed that the positively charged arginine residues counteract the close positioning of two negatively charged groups (the phosphate moiety of N1 and the phosphate ion which attacks the phosphorester linkage at the 3′-terminus) and that they stabilize the transition state.[27] The key residues of the active site are conserved in archaeal exosomes and in bacterial RNase PH and PNPase.[25,27]

However, the proposed general acid/base mechanism cannot fully explain the magnesium ion dependence of RNA degradation by all these enzymes.[14,28] Recently, manganese was identified at the active site of *E. coli* PNPase.[28] Mn^{2+} can substitute for Mg^{2+} and supports catalysis, but is easier identifiable in crystal structures. The metal ion was found to be coordinated by two conserved aspartate residues and a conserved lysine residue and it was proposed that the aspartate residues support catalysis. It was also suggested that the activation of the phosphate for nucleophilic attack on the terminal phosphoester bond is metal dependent and that metal-assisted catalysis is conserved among phosphorolytic RNases including the archaeal exosome (Fig. 1).[28]

The arising rNDP product is not released simply by diffusion, but a conformational change[25] at the N1-binding site leads to controlled (active) release of the product through a conserved side channel[25,27] in concert with the entry of an inorganic phosphate ion. Then, the RNA substrate is translocated in a way that the most 3′-end nucleotide is positioned at the N1-binding site.[25] In a reaction reverse to phosphorolysis, the Rrp41-Rrp42 hexamer polymerises RNA,[9] and the polymerisation seems to follow the steps described above in the opposite direction. Degradation and polymerization (tailing) of RNA are most probably two physiologically important functions of the exosome in Archaea, similar to the functions of PNPase in bacteria and chloroplasts.[11]

Following the path of RNA/protein interaction from an active site at the bottom to the top of the hexamer, the next nucleotide, N5, interacts with two Rrp41-Rrp42 dimers, N7 also interacts with two dimers and N10 interacts with all three dimers at the neck structure near the top of the hexamer (Fig. 1).[25] These interactions are essential for RNA degradation.[25,27] The interaction of N7 and N10 with the exosome is sequence-unspecific but is mediated by the base and not by the phosphate-ribose backbone. The N7 and N10 binding sites are flexible structures of the exosome, which are stabilised upon RNA binding.[25,27]

The neck of 8 to 10 Å in diameter is formed by loops of the Rrp41 subunits and ensures that only single stranded RNA can be threaded into the channel to reach one of the active sites near the bottom of the hexamer.[27] This was demonstrated using RNA oligoribonucleotides containing a stable stem-loop structure at the 5′-end followed by poly(A) tails of different length in degradation assays with the hexameric ring of the *S. solfataricus* exosome. Only substrates carrying tails of at least 10 nt were degraded and a tail of approximately 9 nt remained intact, verifying experimentally that the single stranded RNA substrate follows a path from the top to the bottom of the hexamer, even in the absence of the RNA-binding proteins Rrp4 and Csl4.[27] This can be explained by the electrostatic surface of the hexamer, which is negatively charged at the bottom and on the side (excluding interactions with RNA in these regions) and is positively charged at the entry pore and at the central channel down to the active sites.[10]

The Flexible RNA-Binding Cap

In the nine-subunit form of the archaeal exosome, three polypeptides which can be represented by Rrp41 and/or Csl4, bind on the top of the hexameric ring forming a flat, trimeric cap with a central pore.[10] The trimeric cap (also called the RNA binding ring) contains multiple domains with RNA binding capability: Rrp4 comprises an N-terminal domain and the RNA binding S1 (ribosomal protein S1 homology) and KH (protein K homology) domains, while Csl4 is composed of an N-terminal domain and the RNA binding S1 and Zn-ribbon domains (Fig. 1).[7] Crystallographic studies revealed that the hydrophobic surfaces of the nine domains of the trimeric cap are involved in protein-protein interactions between the individual domains and between the cap and the hexamer. The remaining cap surface represents the top of the nine-subunit exosome and is suitable for interaction with RNA substrates and accessory protein factors.[10]

A comparison of the crystal structures of the two isoforms of the *A. fulgidus* exosome, containing either Rrp4 or Csl4 revealed that in both cases the S1-domains are located at the centre and form a pore which is 15 Å wide (in the case of Rrp4) or 18 Å wide (in the case of Csl4). Although Rrp4 and Csl4 are anchored to the hexameric RPD-ring via their N-terminal domains and their S1-domains form the central entry pore for the substrate,

the positions of the KH and Zn-ribbon domains differ significantly in the two different isoforms. However, their positions do not exclude the existence of heterotrimeric caps, since the individual polypeptides of a cap bind independently to the RPD-hexamer and do not interact with each other. Indeed, it was possible to reconstitute recombinant exosomes with heterotrimeric caps.[10]

The spatial structure of the Rrp4-containing nine-subunit exosome of *S. solfataricus* was determined by two different groups.[18,26] Lorentzen et al resolved the structure of a symmetric protein complex and noticed that the S1 and KH domains interact closely and seem to form a single structural unit. The S1/KH unit possesses higher temperature factors than the well ordered N-terminal domain, indicating that the S1/KH part of Rrp4 is flexible.[18] In the structure resolved by Lu et al, the internal symmetry of the Rrp4-ring was broken by rigid body and thermal motions, although the intermolecular interactions between the Rrp4 ring and the RPD-hexamer of Rrp41 and Rrp42 were similar to the previously described.[26] Each of the Rrp4 subunits was found to possess distinct thermal and conformational characteristics, while the RPD-hexamer was rigid. The major difference to the structure published by Lorenzen et al is the different position of the S1 and KH domains of one of the Rrp4 subunits—these domains are moved away from the central pore, which becomes wider.[26]

These data strongly suggest that the RNA binding ring on the top of the hexamer is a highly flexible structure and several studies support this suggestion. As discussed by Lu et al,[26] major conformational differences between the exosomes of *A. fulgidus* and *S. solfataricus* are observed in the RNA-binding ring. Moreover, the S1 and KH domains of bacterial PNPases are disordered and not visible in the crystal structures, consistent with a high flexibility of these domains.[20,29] Most importantly, the analysis of the *Pyrococcus* exosome in solution by small angle X-ray scattering revealed that the Rrp4 subunits are attached to the hexameric core (presumably by the N-terminal domain) as extended and flexible arms[13] (which probably consist of the S1 and KH domains).

In addition to providing a substrate binding surface, the flexible RNA binding cap also influences the structure of the RPD-hexamer. The shape and the size of the central channel of the hexamer is somewhat different between the two isoforms of the *A. fulgidus* exosome due to differences in the Rrp41 structure.[10] The central channel of the RPD-hexamer of *S. solfataricus* is narrowed in presence of Rrp4, similarly to what was observed for *E. coli* PNPase.[29] Such structural plasticity indicates that the two different RNA binding subunits may allosterically regulate the catalytically active core of the RPD-ring and is compatible with the observation that different isoforms of the exosome differ in their activities.[10,12]

It is important to notice that Rrp4 and Csl4 are conserved in the exosomes of Archaea and Eukarya,[22,30-32] suggesting important differential roles for these proteins, most probably in substrate selection. The two different isoforms of the archaeal exosome (the Rrp4-exosome and the Csl4-exosome) harbor different RNA binding domains[7] and different electrostatic surfaces,[10] consistent with the idea that Rrp4 and Csl4 are responsible for the interaction with different molecules. Indeed, we found that the *S. solfataricus* Rrp4-exosome strongly prefers poly(A), while the Csl4-exosome more efficiently degrades heteropolymeric RNA (Roppelt, V., Klug, G., Evguenieva-Hackenberg, E., submitted).

It is assumed that RNA is bound by the S1-subunits Rrp4 and/or Csl4 and the single stranded 3′-end is threaded through the central channel to reach an active site on the bottom of the hexameric ring (Fig. 1).[18] Although the hexameric ring is sufficient for RNA degradation, the S1-subunits Rrp4 and Csl4 strongly increase RNA binding and

RNA degradation by the exosome.[10,12,13,24] Importantly, highly structured, natural tRNA cannot be degraded by the *S. solfataricus* hexamer, but is easily degraded by the Rrp4- and Csl4 exosomes.[14] Most probably, binding of tRNA by the multiple RNA binding domains at the top surface of the exosome leads to ATP-independent unwinding of secondary structures, a mechanism which was also proposed for degradation of structured RNA by RNase R and the eukaryotic exosome.[33]

PHYSIOLOGICAL FUNCTIONS

The recombinant archaeal exosome is capable to degrade RNA phosphorolytically and to perform the opposite reaction, untemplated synthesis RNA using rNDPs.[9-12,14] These activities are also exhibited by native coimmunoprecipitated exosomes and it was shown that the exoribonucleolytic and the polynucleotidylation activities of the cell-free extract of *S. solfataricus* can be assigned to the exosome.[11,12] Both activities seem to be physiologically relevant, like in the case of PNPases in bacteria and chloroplasts, which acts in vivo as exoribonucleases and as RNA-tailing enzymes.[15,16] RNA tails serve as loading platforms for exoribonucleases and tailed RNAs are thus destabilised and degraded faster than nontailed molecules.[34] Like PNPase, the archaeal exosome synthesizes heteropolymeric RNA-tails.[11,14] Sequences of posttranscriptionally added tails from exosome-containing Archaea were determined and tailed RNAs were identified as truncated mRNA and rRNA molecules. A tailed precursor of 16S rRNA was also detected.[11,35] Since RNA tailing is an event preceding and enhancing RNA decay, these data suggest a function of the exosome in targeting mRNA and rRNA fragments for degradation and in rRNA maturation.[34]

The regulation of the dual function of PNPase and the archaeal exosome is still not clear. It was proposed that local changes in the concentration of inorganic phosphate, rNDPs and Mg^{2+} contribute to this regulation, since such changes modulate the activity in vitro.[14] The reversible phosphorolytic activity probably allows to save energy avoiding the necessity to use rNTPs for synthesis of RNA tails, but the difficulty to regulate the two directions of the reaction is possibly the reason why the eukaryotic nine-subunit exosome has lost its activity and the RNA degradation and polynucleotidylation functions were separated in higher organisms.[36] The eukaryotic nine-subunit exosome is responsible for recruitment of substrates, their unwinding and channelling through the central hole of the hexameric ring, but RNA degradation is performed hydrolytically by Rrp44 which interacts with the bottom of the hexamer.[33,36-38] The untemplated synthesis of short, destabilizing poly(A)-tails to RNA is performed by different protein complexes named TRAMP in eukaryotic cells.[39,40]

Although important and probably essential aspects in RNA processing and degradation depend on the exosome, not all Archaea harbour this protein complex: in most methanogens and in halophilic Archaea, the genes encoding Rrp4, Rrp41, Rrp42 and Csl4 were lost.[7,11,35] In such organisms, posttranscriptionally added RNA-tails are not detectable.[11,35] The correlation of the presence of the exosome with the presence of heteropolymeric RNA tails in Archaea and the lack of genes encoding other polynucleotidylating enzymes in archaeal genomes supports the view that RNA tailing is a major function of the exosome. Interestingly, the exosome-less Archaea still harbour DnaG, suggesting an extraordinarily important role for this protein in RNA metabolism.[34]

CONCLUSION

Although the archaeal exosome shows structural similarity to its eukaryotic counterpart, it is functionally similar to bacterial PNPase. This is in agreement with the structure of mRNA in the third domain of life, which is similar to bacterial and not to eukaryotic mRNA—archaeal mRNA is often polycistronic, does not carry long stabilizing poly(A) tails but short, presumably destabilizing heteropolymeric tails at the 3′-end and is not capped by methylguanosine.[34] Recently we found that the *S. solfataricus* exosome is localized at the cell periphery and cosediments with membranes in sucrose gradients (Roppelt et al, submitted). The localization of the archaeal exosome at the membrane is an additional parallel between the machineries for RNA processing and degradation in Archaea and Bacteria. RNA-degrading protein complexes in *E. coli* and *Bacillus subtilis* were also shown to be membrane-bound,[41-43] suggesting a need of prokaryotic cells to spatially organize RNA processing and degradation.

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NOTE ADDED IN PROOF

The two papers by Roppelt V et al were in press at the publication of this book: (1) Roppelt V, Klug G, Evguenieva-Hackenberg E. The evolutionarily conserved subunits Rrp4 and Csl4 confer different substrate specificities to the archaeal exosome. FEBS Lett. 2010 May 17. [Epub ahead of print]; and (2) Roppelt V, Hobel CF, Albers SV et al. The archaeal exosome localizes to the membrane. FEBS Lett. 2010 May 17. [Epub ahead of print].

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