Rrp4 and Csl4 Are Needed for Efficient Degradation but Not for Polyadenylation of Synthetic and Natural RNA by the Archaeal Exosome[†]

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ABSTRACT: The exosome of the archaeon *Sulfolobus solfataricus* is a protein complex with phosphorolytic and polyadenylating activity. Little is known about its substrates and the regulation of its functions. We characterized the catalytically active hexameric ring composed of *Sso*Rrp41 and *Sso*Rrp42, and the nine-subunit exosomes containing in addition RNA binding protein *Sso*Rrp4 or *Sso*Csl4 under various reaction conditions. The exosome synthesized heteropolymeric RNA tails and exhibited the highest in vitro activity at 60−70 °C. $MgCl_2$ was necessary for exosome activity. The two reactions, degradation and polyadenylation of RNA, were inhibited by increasing glycerol and KCl concentrations but were differently influenced by changes in pH and by increasing $MgCl_2$ concentrations. The three protein complexes with different compositions were similarly influenced by increasing concentrations of glycerol, KCl, and $MgCl_2$, but the *Sso*Rrp4 exosome behaved differently with respect to pH changes. A 20-nucleotide poly(A) tail enabled the degradation and the polyadenylation of a 16S rRNA-derived transcript by the hexamer. Generally, RNA synthesis by the hexamer was more efficient than RNA phosphorolysis. Single-stranded poly(A) RNA, a heteropolymeric 97-nucleotide transcript, and natural tRNA were quickly polyadenylated, showing that these substrates were bound and their 3′-ends reached the active site. Despite this, their efficient degradation was possible only in the presence of *Sso*Rrp4 or *Sso*Csl4. Thus, strong substrate binding by *Sso*Rrp4- or *Sso*Csl4-containing exosomes is more important for phosphorolysis than for tailing of RNA. In summary, the data suggest that subunit composition and Mg^{2+} are involved in the regulation of exosome activity.

The exosome is an essential protein complex originally found in Eukarya, which is involved in exoribonucleolytic processing and degradation in the 3′ to 5′ direction (*1, 2*). The eukaryotic exosome contains six different polypeptides (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, and Mtr3), each with an RNase PH domain (RPD), which form a ring. On the top of the ring, three different polypeptides are bound (Rrp4, Rrp40, and Csl4), which contain RNA binding domains S1 and KH (*3*). Despite the presence of six RPDs, this nine-subunit exosome does not exhibit phosphorolytic activity in vitro. The RNase activity of the eukaryotic exosome is due to the presence of a tenth subunit (Rrp44, also called Dis3) with a hydrolytic activity, which binds to the bottom of the hexameric RPD ring (*3*−*5*). The cytoplasmic and nuclear forms of the exosome contain additional, different subunits and interact with other protein factors, which enable them to fulfill the various physiological functions assigned to the exosome (recently reviewed in refs *2* and *6*).

A similar protein complex with a poorly investigated physiological role was found in Archaea (*7*−*11*). In the archaeal exosome, the hexameric ring is formed by three dimers of the orthologs of Rrp41 and Rrp42 and degrades poly(A) RNA phosphorolytically. Rrp41 harbors the active site, but the ring structure is necessary for activity (*12*). Three polypeptides with RNA binding domains bind on the top of the ring. These polypeptides are the orthologs of Rrp4, which contains an S1 and a KH domain, and/or the ortholog of Csl4, which contains an S1 and a Zn-ribbon domain (*9, 13*). Their presence in the recombinant exosome enhances RNA binding and influences degradation of a short poly(A) RNA substrate by the hexameric ring (*9, 14*). The overall structure of the archaeal nine-subunit exosome is very similar to the nine-subunit exosome of Eukarya and to the bacterial polynucleotide phosphorylase (PNPase) (*2, 3, 9, 12, 13*). The archaeal exosome contains at least one additional subunit with an unknown function, a protein annotated DnaG (*8, 10, 14*).

PNPase is a homotrimer with two RPD domains and an S1 and a KH domain in each monomer. The six RPDs form a ring structure, on the top of which the RNA binding domains are arranged (*15*). Only one of the RPD domains in the monomer of *Escherichia coli* PNPase harbors an active site, while in chloroplast PNPase, both domains show phosphorolytic RNase activity (*15, 16*). The phosphorolysis of RNA and nontemplated RNA synthesis represent the two opposite directions of the same reaction. For PNPase and RNase PH, it was shown that the direction depends on the

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concentrations of nucleoside 5′-diphosphates (NDPs)[1] and phosphate ions in the reaction buffer (*17*−*19*).

It is well established that one of the physiological functions of PNPase in bacteria and organelles is to synthesize heteropolymeric adenine rich RNA tails, which target RNA for degradation (*20, 21*). It was proposed that local changes in the concentration of NDPs and phosphate may regulate the in vivo function of PNPase as an RNase or RNA tailing enzyme (*20*). Heteropolymeric adenine rich RNA tails were also found in archaeal species harboring exosomal genes and were absent from species without the exosome (*22, 23*), and it was shown that the archaeal exosome can synthesize homopolymeric poly(A) tails that elongated a poly(A) substrate in vitro (*12, 22*). These findings support the view that the exosome in Archaea has a dual function: it is an RNA-tailing and RNA-degrading enzyme. In contrast, the eukaryotic exosome itself is not involved in the synthesis of short destabilizing poly(A) tails on RNA, in accordance with its lack of phosphorolytic activity (*3, 4*). However, the RNA degrading activity of the eukaryotic exosome is enhanced in vitro by polyadenylation of the substrate by the TRAMP complex. In vivo, the TRAMP complex polyadenylates RNA molecules that are subsequently degraded by the exosome (*2, 24, 25*). Thus, the three domains of life, Eukarya, Bacteria, and Archaea, exploit similar strategies to ensure efficient RNA degradation in the 3′ to 5′ direction.

We established the thermoacidophile *Sulfolobus solfataricus* as a model organism for investigating the exosome in Archaea. The two activities of the archaeal exosome, RNA degradation and RNA tailing, have not been characterized in detail. Moreover, it was not clear whether RNAs other than poly(A) oligoribonucleotides can be processed by this protein complex and whether exosomal subunits *Sso*Rrp4 and *Sso*Csl4 play a specific role. Our results show that the two activities of the exosome are influenced in a different manner by changes in pH and $MgCl_2$ concentration and that *Sso*Rrp4 and *Sso*Csl4 are important for efficient degradation but not for polyadenylation of RNA.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and DNA Methods. S. solfataricus strain P2 was grown as previously described (*26*). *E. coli* BL21(DE3) and JM 109 were grown on LB medium or on standard I medium (Difco) with 100 μg/mL ampicillin. The pET-MCN plasmids containing the genes encoding *Sso*Rrp41, *Sso*Rrp42, *Sso*Rrp4, and *Sso*Csl4, which we used for protein overexpression, were previously described (*12*−*14*). pSP72 (Promega) was used for runoff in vitro transcription. DNA isolation from *S. solfataricus* was performed by standard procedures (*27*). To amplify the last 600 bp and the last 170 bp of the 16S rRNA gene of *S. solfataricus*, respectively, the following oligonucleotides were used: *Sso*16S-927forward (5′-gtaatacgactcactatagggagcaccacaaggggtg-3)′, *Sso*16S-1370forward (5′-gtaatacgactcactatagggtgaatacgtccctgctcc-3′), *Sso*16S-reverse (5′-ggaggtgatccagccgcag-3′), and *Sso*16S-T_{20}-reverse [5′-$(t)_{20}$ggaggtgatccagccgcag-3′]. The forward nucleotides contain the T7 promoter sequence (underlined). The amplified sequences served as templates for in vitro transcription of 16S rRNA segments, which form discrete domains as judged from the proposed secondary structure of archaeal 16S rRNA (*28*).

Reconstitution of Protein Complexes. The purification of the recombinant subunits of the *S. solfataricus* exosome and the reconstitution of the protein complex were performed essentially as previously described (*12, 14*), using proteins prepared under native conditions or by refolding. When native proteins were used, *E. coli* BL21(DE3) cells transformed with the expression vectors containing the genes for *Sso*Rrp41 and *Sso*Rrp42 (*12*) were grown to an OD_{600} of 0.5, and expression of the proteins was induced overnight at room temperature by adding 0.5 mM IPTG. Ten grams of wet cells was resuspended in 20 mL of 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and opened by sonication. The proteins (N-terminally His tag-fused) were then purified by Ni-NTA affinity chromatography under native conditions. The *Sso*Rrp41−*Sso*Rrp42 complex was reconstituted by mixing 5 mg of *Sso*Rrp41 with 8 mg of *Sso*Rrp42 (molar ratio of approximately 1.5) in a buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 10% glycerol, and 1 mM DTT for 30 min at room temperature and was purified by size exclusion chromatography (HiLoad Superdex 200 16/60 size exclusion chromatography column, Amersham Biosciences, and FPLC System, Pharmacia). Aliquots were stored at −80 °C, and repeated thawing was avoided. The proteins were quantified by the Bradford method and by comparison to suitable dilutions of a BSA standard on silver-stained SDS−PAA gels. For reconstitution of nine-subunit exosomes, the hexameric ring was mixed with equimolar amounts of *Sso*Rrp4 or *Sso*Csl4 in the reaction buffer and incubated for 30 min on ice prior to addition of the substrate. This procedure ensured comparable activities of the hexamer in all reaction mixtures.

When refolding was performed, the procedure described in ref (*14*) was used, with a minor difference: the refolded complexes were incubated at 60 °C instead of 70 °C prior to gel filtration.

RNA Methods. For 5′-end labeling, 10 pmol of the poly(A) oligoribonucleotide (30-mer; CureVac, Tübingen, Germany) or 10 pmol of total RNA from *Saccharomyces cerevisiae* (Sigma) was incubated with T4 polynucleotide kinase (NEB or Fermentas) and 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in a 10 μL reaction mixture containing the buffer supplied by the manufacturer for 30−60 min at 37 °C. The reaction was stopped with 40 μL of STE buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA], and the unincorporated nucleotides were removed using MicroSpin G-25 columns (GE Healthcare). In vitro transcription using T7 RNA polymerase and purification of [α-^{32}P]UTP-labeled transcripts on denaturing gels were performed as previously described (*29, 30*). The final concentrations of the ribonucleoside triphosphates (rNTPs) were as follows: 0.5 mM rATP, 0.5 mM rGTP, 0.5 mM rCTP, and 0.1 mM rUTP (lowU-mix); 20 μCi of [α-^{32}P]UTP (3000 Ci/mmol) and T7 RNA polymerase (NEB) were added in a final volume of 20 μL. The in vitro transcription was performed for 2−4 h at 37 °C.

A typical 10 μL degradation or polyadenylation assay was performed with 1000−5000 cpm substrate in a thermocycler. The originally used assay buffer contained 20 mM HEPES

[1] Abbreviations: GuHCl, guanidine hydrochloride; nt, nucleotide(s); NDPs, nucleoside 5′-diphosphates; P_i, inorganic phosphate; PAA, polyacrylamide.

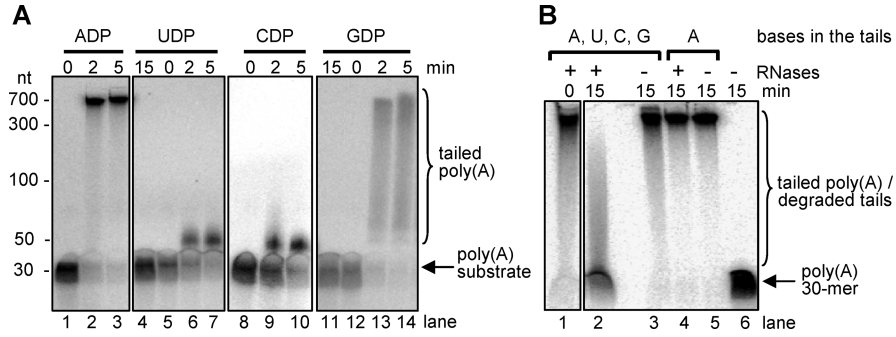

FIGURE 1: RNA synthesis by the *Sso*Rrp41−*Sso*Rrp42 protein complex (hexamer) in vitro. Production of tails comprising different bases. Phosphorimages of 10% denaturing PAA gels. (A) The hexamer synthesizes different homopolymeric tails using 5′-end-labeled 30-mer poly(A) RNA as the primer substrate. Different nucleoside 5′-diphosphates (ADP, UDP, CDP, or GDP, 10 mM each, indicated above the panel) were added to the reaction buffer, which contained 20 mM HEPES (pH 7.9), 60 mM KCl, 13.4% glycerol, 5 mM $MgCl_2$, 375 mM trehalose, 0.1 mM EDTA, and 2 mM DTT. The incubation time (minutes) at 60 °C is indicated above the panel. In lanes 4 and 11, negative controls, the substrate was incubated in the buffer without protein. The migration behavior of the RNA Century Marker is indicated. (B) RNase degradation assays with RNA tails synthesized by the hexamer when all four nucleoside 5′-diphosphates were present together in the polynucleotidylation mixture (lanes 1−3) or when only ADP was used (lanes 4 and 5). Only the loaded lanes are numbered; there is an empty lane between the loaded lanes 2 and 3. The incubation time (minutes), the presence or absence of RNases in the reaction mixture, and the base composition of the RNA tails [heteropolymeric tails presumably containing all four bases or homopolymeric poly(A) tails] are indicated above the panel. In lanes 3 and 5, negative controls, RNases A and T1 were not added. Lane 6 shows the 5′-end-labeled poly(A) RNA used as the primer substrate in the polynucleotidylation reaction. The reaction buffer is described for panel A.

(pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 12% glycerol, and 375 mM trehalose; buffer variations are described in the text and in the figure legends. Approximately 0.6 pmol of each protein complex (approximately 60 ng of each subunit of the exosome) was used in each reaction mixture, to which approximately 6 fmol RNA was added. Variations in the amount of the substrate, incubation time, and incubation temperatures are indicated.

Control assays with *E. coli* lysates were performed as follows. For the *E. coli* extract, 1 mL of overnight culture of *E. coli* BL21(DE3) was pelleted at 6000*g* and 4 °C, dissolved in 1 mL of lysis buffer used for isolation of the recombinant exosomal subunits, and opened by sonication. Cell debris was removed by centrifugation at 12000*g* at 4 °C, and 2 μL of the supernatant (crude extract) was used in reaction mixtures with a final volume of 10 μL. The assays were assembled on ice in 0.5 mL tubes and then incubated at 60 or 37 °C in a thermal cycler. As indicated, in some cases the *E. coli* extract and the *S. solfataricus* hexamer were preincubated in the reaction buffer for 20 min at 60 °C. Then, the RNA substrate was added, and the reaction mixture was incubated further at 60 °C.

The reaction mixtures were separated in denaturing polyacrylamide gels (8, 10, or 18%). TLC analysis was performed as described in ref (*14*). Briefly, 6 μL of the reaction mixture was spotted on a PolygramCEL 300 PEI TLC plate (Machery&Nagel). After the sample had been washed with methanol, chromatography was performed with 0.9 M GuHCl (pH 6.3). Substrate and degradation products were analyzed by autoradiography. To obtain UDP and UMP or AMP and ADP standards, the RNA substrates were incubated with a PNPase-containing fraction from *E. coli* (producing nucleoside 5′-diphosphates in the presence of P_i) or an RNase R-containing fraction from *Pseudomonas syringae* (producing nucleoside 5′-monophosphates), respectively (*31*). Signals were detected and quantified using a Bio-Rad molecular imager and Quantity One (Bio-Rad).

RNA secondary structures of short in vitro transcripts were predicted using the homepage of M. Zuker and *mfold* (*32*).

Hazardous Procedures. The use of radioactively labeled nucleotides and RNA molecules should be performed using adequate equipment and according to safety rules.

RESULTS

The S. solfataricus Exosome Synthesizes Heteropolymeric RNA Tails. The exosome was proposed to be the enzymatic activity that synthesizes heteropolymeric adenine rich RNA tails without a template in Archaea (*22, 23*). To analyze in vitro the capability of the exosome to produce RNA tails containing different bases, we first tested whether the *Sso*Rrp41−*Sso*Rrp42 complex can use separately the four nucleoside 5′-diphosphates (NDPs), with poly(A) as the primer substrate. The longest tails, longer than 700 nt, were produced in the presence of ADP (Figure 1A, lanes 1−3). When UDP or CDP was used, short tails of approximately 20 nt were produced (lanes 5−7 or 8−10, respectively). The use of GDP resulted in the synthesis of tails with very different lengths, and the reaction products appeared as a smear (lanes 12−14). Thus, the hexameric ring can synthesize homopolymeric RNA consisting of each of the four different bases, with the following order of preference for addition: A > G > U/C.

In the next experiment (Figure 1B), the four nucleoside 5′-diphosphates (NDPs), with poly(A) as the primer substrate, were used together in a tailing reaction and the resulting tails (lane 1) were treated with RNase A and RNase T1 (lane 2). In parallel, homopolymeric poly(A) tails were synthesized and treated with the two RNases (lane 4). RNase A cleaves preferentially between a pyrimidine and A, and RNase T1 cleaves 3′ of G residues. Therefore, the two RNases were used to discriminate between homopolymeric poly(A) tails and heteropolymeric tails. The tails produced in the presence of all four NDPs were degraded by the RNases, while the homopolymeric poly(A) tails remained intact (Figure 1B). This result shows that the hexameric ring is capable of synthesizing heteropolymeric RNA tails using NDPs. The nine-subunit exosomes containing *Sso*Rrp4 or *Sso*Csl4 also synthesized heteropolymeric RNA tails (not shown). How-

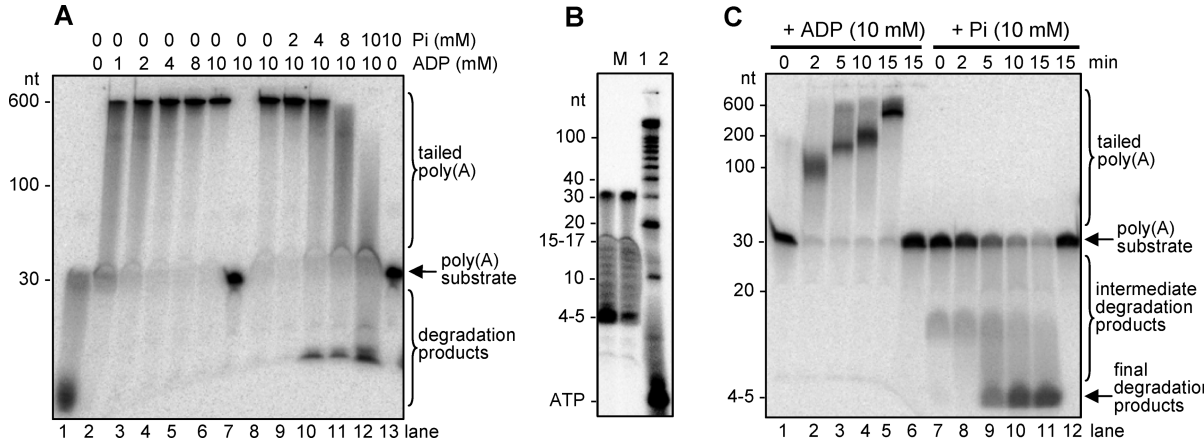

FIGURE 2: Concentrations of ADP and P_i determine whether the hexameric core of the exosome synthesizes or degrades RNA in vitro. Phosphorimages showing the results of polynucleotidylation and degradation assays with the hexamer and 5′-end-labeled 30-mer poly(A) RNA at 60 °C. The reaction buffer is described in the legend of Figure 1A. (A) Denaturing PAA gel (10%). The concentration of ADP and/or P_i (K_2HPO_4) added to the reaction mixture is indicated above the panel. The incubation time was 10 min. In lanes 7 and 13, negative controls, the substrate was incubated in the buffer without addition of protein. The migration behavior of molecules of marker molecules (600 and 100 nt) is indicated. (B) Denaturing PAA gel (18%). M, RNA Decade Marker (Ambion). Free ATP used for the labeling of the marker is detectable. Lanes 1 and 2 show the incubation of the substrate for 3 and 6 min, respectively. P_i at 10 mM was added. (C) Denaturing PAA gel (10%). The concentration of ADP and/or P_i added to the reaction mixture and the incubation time (minutes) are indicated above the panel. The migration behavior of marker molecules (nt) is indicated. Lanes 6 and 12 were negative controls.

ever, the exosome could not use NTPs instead of NDPs for RNA synthesis (not shown), although it was reported that chloroplast PNPase can tail RNA when NTPs are added instead of NDPs to the reaction mixture (*21*).

Influence of P_i and NDPs on Exosome Activity. The phosphorolysis of RNA using inorganic phosphate (P_i) ions and RNA synthesis using NDPs represent the two directions of a reversible reaction catalyzed by RNase PH and the RNase PH-like core of the prokaryotic PNPase and of the archaeal exosome (*12, 17*−*19, 21, 22*). It is known that the concentrations of P_i and NDPs determine the direction of the reaction performed by RNase PH or PNPase (*17*−*19*). The archaeal exosome is still not characterized with respect to the factors which regulate its degradative and synthetic functions. We tested the activity of the hexameric ring of the *S. solfataricus* exosome at different concentrations of ADP and P_i using the 30-mer poly(A) substrate. Figure 2A shows increasing polyadenylation activity with an increase in ADP concentration from 1 to 10 mM (lanes 2−6). In the presence of 10 mM ADP and increasing concentrations of P_i (K_2HPO_4) up to 10 mM, the polyadenylation reaction was suppressed and the degradation activity was increased (lanes 8−12). The hexameric ring degraded the substrate without addition of P_i to the buffer (Figure 2A, lane 1). This degradation is not due to hydrolytic activity but rather to the presence of phosphate in the protein fractions (see Figure 5D,E and the corresponding text below).

According to the migration behavior in high-resolution polyacrylamide gels, the size of the final degradation products was 4−5 nt (Figure 2B). Molecules shorter than 15−17 nt were degraded slowly and most probably in a distributive manner, leading to the occurrence of detectable intermediate products. In contrast, distinct degradation products with lengths between 30 and 17 nt were not detected, suggesting that RNA in this length range was degraded processively (Figure 2B).

When we added 10 mM ADP (for polyadenylation) or 10 mM P_i (for degradation) to the reaction mixture and compared the time course of polyadenylation to the time course of degradation of RNA, it was obvious that the polyadenylation reaction was more efficient (Figure 2C). In the polyadenylation assay, essentially all substrate molecules (97%) were bound and were elongated after incubation for 2 min (compare lane 1 to lane 2), and the tails became longer with an increase in incubation time (lanes 3−5). Thus, the exosomal ring was capable of binding and processing the substrate efficiently under these conditions. In contrast, 86% of the substrate remained intact after incubation for 2 min in the degradation assay (compare lane 7 to lane 8). During further incubation, the amount of intact substrate decreased to 12% (lane 11), but the degradation was clearly slower than the polyadenylation. This may be due to the reaction buffer (the originally used buffer is described in the legend of Figure 1A), which may be optimal for the one direction of the reaction. It is also conceivable that the S1 subunits of the exosome are needed for efficient degradation, while the hexamer is sufficient for polyadenylation, or that the poly(A) RNA is a good substrate for tailing but not for degradation. To address the first two possibilities, we varied the concentrations of different buffer components and tested the polyadenylation and degradation activity of the hexameric ring and of the nine-subunit exosomes containing *Sso*Rrp4 or *Sso*Csl4 using the poly(A) RNA as a substrate.

Influence of Trehalose, Glycerol, and KCl on Exosome Activity. We found that glycerol and trehalose, a natural osmolyte for *S. solfataricus* (*33*), are not needed for degradation and tailing of RNA. The absence or presence of trehalose at concentrations up to 600 mM had no effect on the two reactions (not shown), while increasing glycerol concentrations decreased the degradation and polyadenylation activity, although to a different extent (Figure 1 of the Supporting Information).

An increase in glycerol concentration inhibited RNA degradation by all three complexes. When the glycerol concentration in the samples was increased from 0 to 20%, the amount of substrate remaining intact after incubation with the hexamer increased from 17 to 61% and the amount of final degradation products in the range of 4−5 nt decreased

from 23 to 1%. When the nine-subunit exosomes were used and no glycerol was added, nearly no intact substrate was detected and approximately 40% of the substrate was degraded to oligonucleotides of 4−5 nt. In the presence of 20% glycerol, 2% of the substrate remained intact and only 3−4% of the substrate was degraded to 4−5 nt. Thus, the negative influence of glycerol on the accumulation of final degradation products was similar for all three complexes. However, the effect of the increasing glycerol concentration on the amount of intact substrate still present after incubation was very strong for the hexamer (61% intact substrate at 20% glycerol) and almost negligible for the *Sso*Rrp4- and *Sso*Csl4-containing complexes (2% intact substrate at 20% glycerol) (Figure 1A,B of the Supporting Information). This suggests that the nine-subunit exosomes are very efficient in initiating degradation, leading to the fast disappearance of the substrate band, while the hexamer performs this step inefficiently.

The effect of the S1 subunits of the exosome on polyadenylation was modest (Figure 1C,D of the Supporting Information). Differences in the amount of remaining substrate and the somewhat slower polyadenylation by the hexamer (leading to the detection of shorter reaction products) in comparison to the *Sso*Rrp4 and *Sso*Csl4 exosomes were detected only when very short incubation times of <1 min were applied.

On the basis of the results given above, glycerol and trehalose were omitted from all subsequent assays. The influence of KCl on the degradation and polyadenylation activity of all complexes was similar to that of glycerol: KCl inhibited the activity of the exosome when present at concentrations higher than 300 mM (Figure 2 of the Supporting Information). The KCl concentration used so far (60 mM) led to satisfactory results and was not changed.

Influence of pH and Polyamines on Exosome Activity. Changes in pH influenced the two reactions and the complexes of different compositions in a different manner (Figure 3 of the Supporting Information). Optimal degradation was achieved at physiologically relevant (*34*) pH values of 6 and 7. Increasing the pH to 9 resulted in a decreasing degradation efficiency. At pH 9, the strongest inhibitory effect was observed for the hexamer, followed by the *Sso*Csl4 complex and the *Sso*Rrp4 complex (Figure 3A,B of the Supporting Information). It should be noted that at pH 9, only 62% of the substrate remained intact in the negative control sample (Figure 3A of the Supporting Information, lane 13). In contrast, the highest polyadenylation activity of the hexamer and of the *Sso*Csl4 complex was observed at pH 8. The polyadenylation activity of these complexes was inhibited at pH 6 and 9. The polyadenylation activity of the *Sso*Rrp4 complex was not dependent on pH differences between pH 6 and 8, and only slight inhibition was observed at pH 9 (Figure 3C,D of the Supporting Information).

It is known that polyamines bind RNA and thus they may mediate interactions between proteins and RNA. We asked whether in the presence of polyamines, the hexamer or the *Sso*Csl4 exosome will exhibit a similar independence with respect to pH like the *Sso*Rrp4 complex. We tested the polyadenylation activity of the complexes in the presence of any of the three polyamines, ornithine, putrescine, and spermidine, at different concentrations. At pH 6, ornithine and putrescine improved the polyadenylation activity of the *Sso*Csl4 exosome (short tails of approximately 20 nt were synthesized) but not of the hexamer (not shown). The polyamine spermidine had the strongest positive effect: at pH 6−8 and in the presence of 3 mM spermidine, the polyadenylation activity of the hexamer and the *Sso*Csl4 exosome was comparable to the activity of the *Sso*Rrp4 exosome (Figure 4 of the Supporting Information). Obviously, the interaction of the substrate with the hexamer and the *Sso*Csl4 exosome in the presence of spermidine is strong enough to enable polyadenylation at physiologically relevant pH values of 6−7.

Influence of $MgCl_2$ and Temperature on Exosome Activity. When $MgCl_2$ was omitted from the buffer, the exosome did not exhibit any activity in the degradation or polyadenylation assays (Figure 5 of the Supporting Information, lanes 2, 8, and 13). Changes in $MgCl_2$ concentration influenced the two reactions in a different manner. An increase in the $MgCl_2$ concentration up to 10 mM led to an increase in the degradation activity of all three complexes (Figure 5A,B of the Supporting Information). Efficient degradation to final products of 4−5 nt was achieved at a minimal $MgCl_2$ concentration of 6 mM. In this respect, the highest activity was shown by the *Sso*Rrp4-containing exosome, which converted 55% of the substrate to 4−5 nt products, followed by the *Sso*Csl4 exosome (35%) and the hexameric ring (18%). However, at 0.8 mM $MgCl_2$, approximately 44% of the substrate remained intact when incubated with the hexamer, in comparison to only 3−4% of intact substrate which can be detected after incubation with the nine-subunit exosomes. Similar results were obtained in the presence of 2 mM $MgCl_2$. It should be noted that 92% of the substrate remained intact in the negative control sample without addition of $MgCl_2$, while in the negative control sample at 10 mM $MgCl_2$, half of the substrate was degraded. These results show that at low $MgCl_2$ concentrations, the nine-subunit exosomes are much more efficient than the hexamer in interacting with the substrate and performing first degradation steps, although all three complexes are rather inefficient in their production of short oligonucleotides under these conditions.

For all three protein complexes, maximal polyadenylation activity was observed at $MgCl_2$ concentrations between 2 and 6 mM, as judged from the length of the reaction products: depending on the composition of the protein complexes and the reaction conditions, between 40 and 80% of the synthesized poly(A) tails were longer than 600 nt. An increase in the $MgCl_2$ concentration to 10 mM led to decreased polyadenylation activity: only 6−20% of the synthesized tails were longer than 600 nt, and the majority of the reaction products had a length between 100 and 600 nt (Figure 5C,D of the Supporting Information). A $MgCl_2$ concentration of 6 mM was suitable for degradation and for polyadenylation assays, and this concentration was used routinely in the subsequent experiments. Cases in which different $MgCl_2$ concentrations were used are identified explicitly.

On the basis of the finding that different $MgCl_2$ concentrations are needed for optimal degradation or polyadenylation activity, we speculated that changes in the concentration of $MgCl_2$ in addition to the concentrations of NDPs and of P_i may regulate the dual function of the archaeal exosome. Figure 3 demonstrates that a change in the concentration of

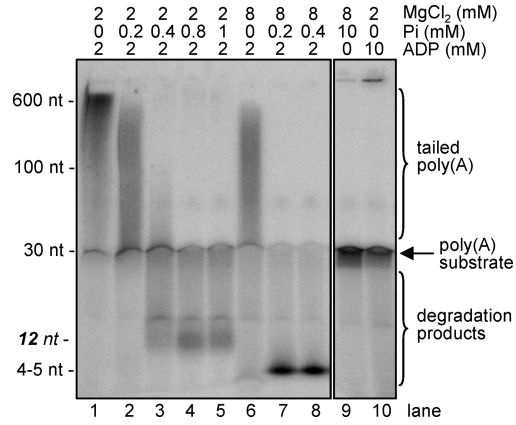

FIGURE 3: Effect of P_i and Mg^{2+} on degradation of poly(A) RNA by the hexameric core of the *S. solfataricus* exosome at a constant ADP concentration. Phosphorimage of a 10% denaturing PAA gel showing the results of polynucleotidylation and degradation assays with the *Sso*Rrp41−*Sso*Rrp42 protein complex and 5′-end-labeled 30-mer poly(A) RNA for 5 min at 60 °C. The reaction buffer contained 20 mM HEPES (pH 7.9), 60 mM KCl, 0.1 mM EDTA, 2 mM DTT, and $MgCl_2$, ADP and P_i (K_2HPO_4) were present at the concentrations indicated above the panel. Lanes 9 and 10 were negative controls.

$MgCl_2$ changes the ratio of ADP to P_i, at which the degradative activity of the hexamer exceeds its polyadenylation activity. The poly(A) substrate was polyadenylated when 2 mM ADP, 0.2 mM P_i, and 2 mM $MgCl_2$ were added to the buffer (lane 2). In contrast, at 8 mM $MgCl_2$ and the same ADP and P_i concentrations, the substrate was degraded to completion (lane 7). Moreover, at 2 mM $MgCl_2$, 2 mM ADP, and 0.8−1 mM P_i, the substrate was not degraded to end products of 4−5 nt, but degradation products with an approximate length of 12 nt accumulated (lanes 4 and 5).

After optimizing the conditions for the two directions of the reaction catalyzed by the archaeal exosome, we performed polyadenylation and degradation assays at different temperatures between 4 and 80 °C. The highest activity of the recombinant *S. solfataricus* exosome was observed under physiologically relevant temperatures between 60 and 80 °C (Figure 6 of the Supporting Information). As before, all subsequent assays were performed at 60 °C.

We excluded the fact that contaminating proteins originating from the expression host *E. coli* are responsible for the observed activities. When *E. coli* cell-free extracts were used in the polyadenylation assays instead of the exosome preparations, no polyadenylation acivity was detected (Figure 7A of the Supporting Information). This shows that *E. coli* enzymes known to use NDPs for polyadenylation (PNPase and RNase PH) did not polyadenylate RNA under the conditions applied in our assays. Additionally, *E. coli* RNases in the cell-free extract were readily inactivated at 60 °C, while the recombinant hexameric ring of the *S. solfataricus* exosome remained stable (Figure 7A,B of the Supporting Information). These results demonstrate that the preparation of the hexameric ring is free of contaminating activities. To show that the increased degradation activity of *Sso*Rrp4- and *Sso*Csl4-containing complexes is not due to contaminating RNases, the used *Sso*Rrp4 and *Sso*Csl4 preparations were also tested. The poly(A) RNA and a synthetic heteropolymeric transcript were not degraded by those protein fractions (Figure 7C of the Supporting Information).

The Poly(A) Tail Enables Polyadenylation and Degradation of a 16S rRNA-Derived Transcript by the Hexameric Ring. Previously, we have shown that an internally labeled transcript of 217 nt, which was derived from *Rhodobacter capsulatus puf* mRNA, was degraded very inefficiently by cell-free extracts of *S. solfataricus* (*14*), raising the question of whether the archaeal exosome can degrade natural RNA. To answer this question, we first decided to use transcripts corresponding to natural substrates of the *S. solfataricus* exosome. The heteropolymeric RNA tails in *S. solfataricus* are obviously products of the exosome, because such tails were detected only in exosome-containing Archaea. 16S rRNA of *S. solfataricus* was found to be one of the tailed RNAs (*22, 23*), and we concluded that 16S rRNA is a substrate of the exosome. Poly(A) rich tails were detected at 3′-ends of 16S rRNA degradation products and of full-length molecules (*22*). Therefore, we synthesized in vitro two internally labeled transcripts, which correspond to the last 600 nt and to the last 170 nt of 16S rRNA of *S. solfataricus*, respectively. The transcripts were used in assays with the hexameric ring as well as with complexes containing *Sso*Rrp4 and *Sso*Csl4. However, we did not detect any products of degradation or polyadenylation reactions (not shown). Then we synthesized a modified form of the 170 nt 16S rRNA-derived transcript with 20 adenylates at the 3′-end. This polyadenylated transcript was tailed and degraded by the hexameric ring of the exosome (Figure 4), showing that a poly(A) tail is helpful for the successful interaction between the substrate and the hexamer.

SsoRrp4 and SsoCsl4 Are Needed for Efficient Degradation but Not for Polyadenylation of Short Heteropolymeric RNAs. Another substrate we used was derived from the multiple cloning site of plasmid pSP72 [MCS-RNA (Figure 5A)]. This short (97 nt) internally labeled in vitro transcript was efficiently polyadenylated by the hexameric ring. The polyadenylation efficiency of the nine-subunit exosomes was similar (Figure 5B). The degradation by the hexamer was also successful (Figure 5C), although it was slower in comparison to degradation of poly(A) RNA. Two intermediate degradation products with sizes of 90 and 64 nt were observed in an 8% PAA gel, which corresponds to the halt of degradation. The result of this size determination fits well to the mechanism for RNA degradation by the hexamer, which should leave a single-stranded region of at least 9 nt when secondary structure is encountered (Figure 5A; (*35*)).The exoribonucleolytically released products were analyzed in a high-resolution 18% PAA gel and by thin layer chromatography (TLC) and were identified as NDPs (Figure 5D,E).

As controls for the TLC analysis we loaded samples in which the MCS RNA was degraded by the *E. coli* degradosome [contains PNPase and produces nucleoside 5′-diphosphates in the presence of P_i (*18, 36*)] or by the *Ps. syringae* degradosome [contains RNase R and produces nucleoside 5′-monophosphates without addition of P_i (*31*)] available in our laboratory (*14*). Lane 2 in panel D and lane 4 in panel E show that NDPs were released even without addition of P_i to the reaction mixtures. This confirmed that RNA degradation under these conditions was due to small amounts of P_i already present in the reaction mixtures and that no alternative degradation mechanism in the absence of phosphate was involved.

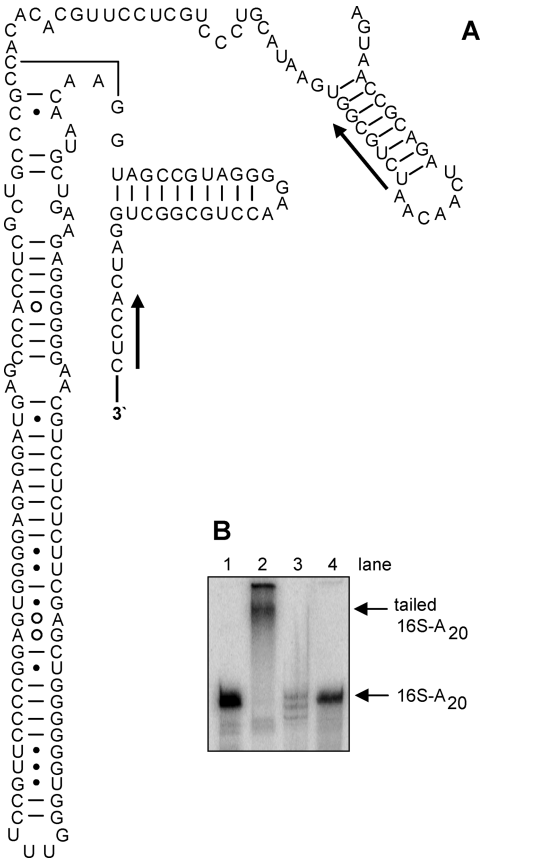

FIGURE 4: 16S rRNA-derived in vitro transcript carrying 20 adenylates at the 3′-end tailed and degraded by the hexameric core of the exosome. (A) Section of the proposed secondary structure of 16S rRNA of *S. solfataricus* (*28*) including the sequence of the 170 nt in vitro transcript used here. The position of 5′-end of the primers used for amplification of the corresponding 16S rDNA part is indicated with arrows. (B) Phosphorimage of a 10% denaturing PAA gel. The input substrate (lane 1) was incubated for 10 min with the hexamer in the presence of 10 mM ADP (lane 2), for 30 min with the hexamer in the presence of 10 mM P_i (lane 3), or for 30 min without protein in the presence of 10 mM P_i (lane 4). The reaction buffer contained 20 mM HEPES (pH 7.9), 6 mM $MgCl_2$, 60 mM KCl, 0.1 mM EDTA, 2 mM DTT, and ADP or P_i as indicated. All incubations were performed at 60 °C.

Further, we found that MCS RNA is degraded much faster by the nine-subunit exosome than by the hexamer (Figure 5F,G). In the high-resolution gel shown in Figure 5F, the bands corresponding to the intact transcript and the 90 nt intermediate product were not well resolved. This intermediate is visible only in lanes 5 (after incubation for 30 min with the hexamer) and 9 (after incubation for 5 min with the *Sso*Csl4 complex) just below the substrate band. The intermediate product of 64 nt was produced by all complexes. With a period of degradation by the hexamer between 5 and 30 min, the amount of intact substrate per lane decreased from 58 to 30%. However, it should be noted that at time zero and in the negative control, only 62−64% of the radioactivity in the sample corresponded to the intact substrate (Figure 5F, lanes 1 and 12; see also the corresponding quantification data in Figure 5G). With a period of degradation by the *Sso*Rrp4 exosomes between 5 and 30 min, the amount of intact substrate decreased from 20 to 3%. Similar values were obtained for the *Sso*Csl4 complex (a decrease from 32 to 4%). Thus, MCS RNA was degraded better by the nine-subunit complexes than by the hexamer, although the polyadenylation efficiency of the three complexes of different composition was comparable.

It is known that RNase PH binds tRNA and is involved in tRNA 3′-end maturation in bacteria (*37*). Recently, it was shown that the eukaryotic exosome degrades hypomodified tRNA after its polyadenylation by other proteins (*25, 38*). To test whether the hexameric ring of the *S. solfataricus* exosome is capable of degrading or polyadenylating natural tRNA, we used 5′-end-labeled tRNA from *Sa. cerevisiae*. The assays were performed at 37 and 60 °C (Figure 6A,B). Polyadenylation of tRNAs by the hexamer was much more efficient than degradation. Approximately half of the tRNAs were polyadenylated after incubation for 20 min at 37 °C, and essentially all tRNAs (95%) were polyadenylated at 60 °C; on the other hand, the amount of intact substrate was not strongly decreased during degradation. Only 1−2% of the substrates were degraded at 37 °C (decrease in the amount of intact substrates per lane from 85% at time zero to 82% at 20 min). More degradation products were detected after incubation for 20 min at 60 °C (40%) than at 37 °C (18%; however, this was comparable to that at time zero). The size of the final degradation products is 4−5 nt (experimental determination not shown).

The three protein complexes of different composition polyadenylated tRNA efficiently (Figure 6C). In contrast to the hexamer, the nine-subunit exosomes degraded tRNA with high efficiency (Figure 6D,E). After incubation for 10 min at 60 °C, only 25−28% of the substrate remained intact in the samples containing nine-subunit complexes, while 58% of the substrate remained intact in the sample containing the hexamer. In comparison, 93% of the substrate was intact in the negative control lane. Careful inspection of Figure 6C revealed that the two nine-subunit exosomes with different compositions produce different intermediate degradation products, which migrate in the gel at positions marked by arrows at the right side of panel. This points to differential interaction of the Rrp4 and Csl4 orthologs with RNAs present in the heterogeneous mixture of molecules used as the substrate in this experiment.

The hexamer of *Sso*Rrp41 and *Sso*Rrp42, which was used for the reconstitution of nine-subunit exosomes and for the activity assays described above, was prepared for previous studies (*14, 22*). To confirm the statement that for efficient degradation of tRNA, the S1 domain-containing subunits of the exosome are needed, we repeated key experiments using independently reconstituted protein complexes. Degradation assays with 5′-end-labeled tRNA confirmed the importance of *Sso*Rrp4 and *Sso*Csl4 for degradation of natural, heteropolymeric RNA (Figures 8−11 of the Supporting Information).

DISCUSSION

This work was performed to gain insight into the functions of the archaeal exosome and its regulation. The physiological temperature optimum for *S. solfataricus* is above 80 °C. However, the in vitro reaction optimum for the recombinant exosome was at 60−70 °C, and lower activity was detected at 80 °C (Figure 6 of the Supporting Information). Most probably, this was due to the lack of additional subunits, which are present in vivo in the *S. solfataricus* exosome (*8, 14*), or of additional cellular factors like chaperones. The ability

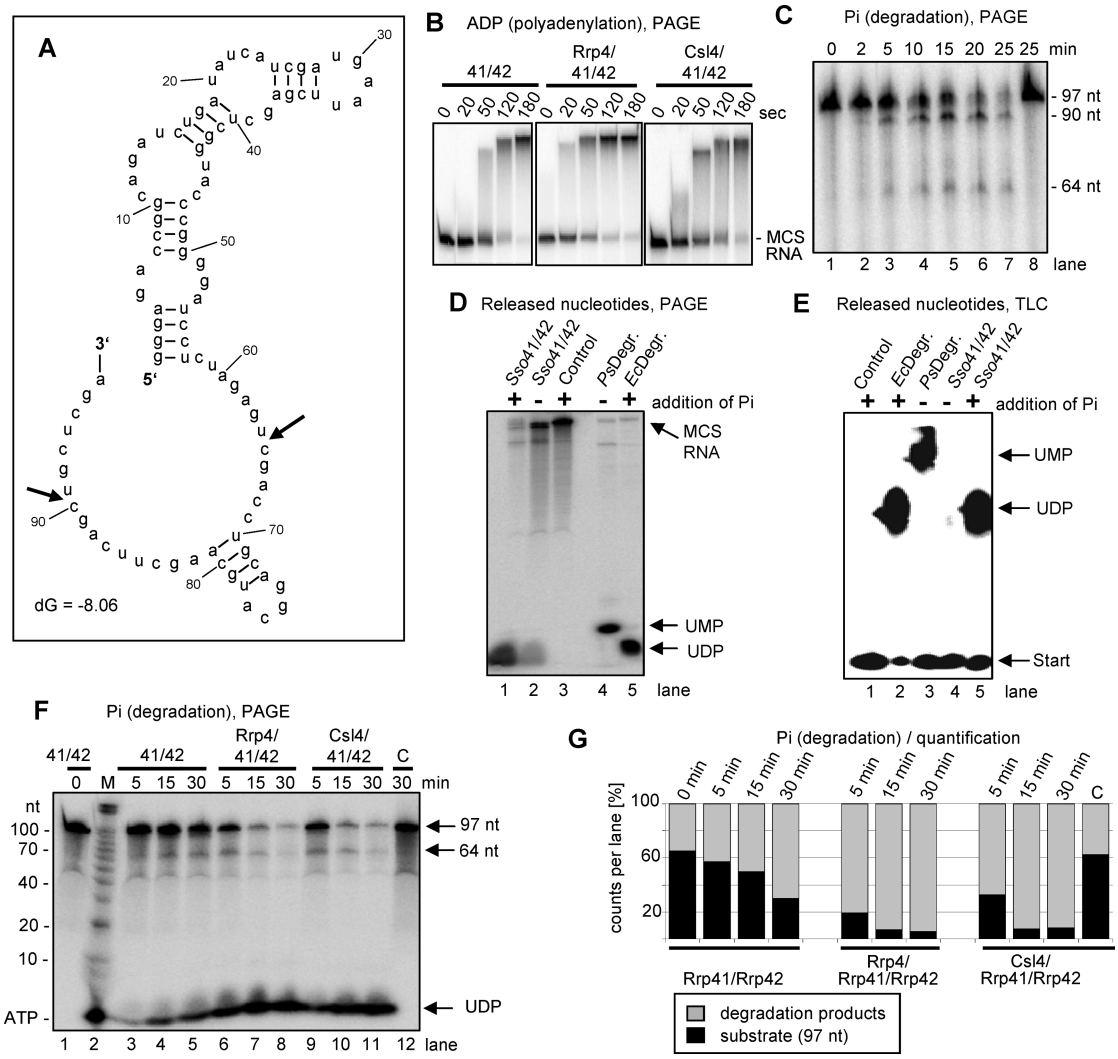

FIGURE 5: Degradation and polyadenylation of a short internally labeled in vitro transcript (MCS RNA) by the hexameric core of the exosome (*Sso*Rrp41/*Sso*Rrp42) and by nine-subunit exosomes (*Sso*Rrp4/*Sso*Rrp41/*Sso*Rrp42 and *Sso*Csl4/*Sso*Rrp41/*Sso*Rrp42, respectively). (A) Predicted secondary structure of the used RNA substrate [*mfold* (*31*)]. The arrows indicate 3′-ends which correspond to the 90 and 64 nt degradation products detected in panel C. (B) Phosphorimage of a 10% denaturing PAA gel showing polyadenylation of MCS RNA by the protein complexes in the presence of 10 mM ADP. The incubation time at 60 °C and the migration behavior of marker molecules (nt) and substrate (MCS RNA) are indicated. (C) Phosphorimage of an 8% denaturing PAA gel showing degradation of MCS RNA by the hexamer. Incubation at 60 °C for the indicated time (minutes). The substrate (97 nt) and two intermediate products (90 and 64 nt) are marked. (D) Phosphorimage of an 18% denaturing PAA gel showing degradation assays with the hexamer (*Sso*41/*Sso*42) for 25 min at 60 °C, with the *Ps. syringae* degradosome (*Ps*Degr.) for 15 min at 20 °C, and with the *E. coli* degradosome (*Ec*Degr.) for 15 min at 37 °C. The control sample was incubated for 25 min at 60 °C. Addition of P_i (+) or usage of buffer without addition of P_i (−) and migration behavior of the substrate and the released nucleosides are indicated. (E) TLC analysis of the samples shown in panel D. (F) Phosphorimage of an 18% denaturing PAA gel showing degradation of MCS RNA by the protein complexes in the presence of 10 mM P_i. Incubation at 60 °C for the indicated time (minutes). Migration behavior of substrate (97 nt) and products (64 nt intermediate and released UDP) is indicated. (G) Quantitative representation of the results shown in panel F. The radioactivity per lane was set to 100%. The composition of the reaction buffer is given in the legend of Figure 4. Lane C was a negative control.

of the *S. solfataricus* exosome to synthesize heteropolymeric RNA tails is consistent with its proposed role as a polynucleotidylation machine in exosome-containing Archaea (*22, 23*). Thus, the archaeal exosome shows not only structural but also functional similarity to the prokaryotic PNPase, which is the enzyme responsible for synthesis of heteropolymeric RNA tails in bacteria and in organelles, in addition to its role as a phosphorolytic 3′ to 5′ exoribonuclease (*20, 21*). This distinguishes it from the eukaryotic exosome, although the two protein complexes possess a nine-subunit core with a high degree of structural similarity (*3, 12*).

Generally, the hexameric ring of the *S. solfataricus* exosome performs RNA polyadenylation much more efficiently than RNA degradation (for an example, see Figure 2C). On the basis of similar RNA degradation assays, we previously suggested that only a minor fraction of the hexameric complexes, which were present in 100-fold molar excess over the substrate, was active in vitro, and we observed a turnover of the substrate by active exosomes (*14*). This interpretation is questioned by the results of the polyadenylation assays shown here. The comparison of the polyadenylation and degradation of poly(A) RNA under the initially applied conditions [13% glycerol, 5 mM $MgCl_2$, and pH 7.9 (Figure 2C)] suggests that the recombinant hexamers are active but synthesize RNA much faster than they are able to degrade RNA. This raised the question of which factors are responsible for these differences: reaction

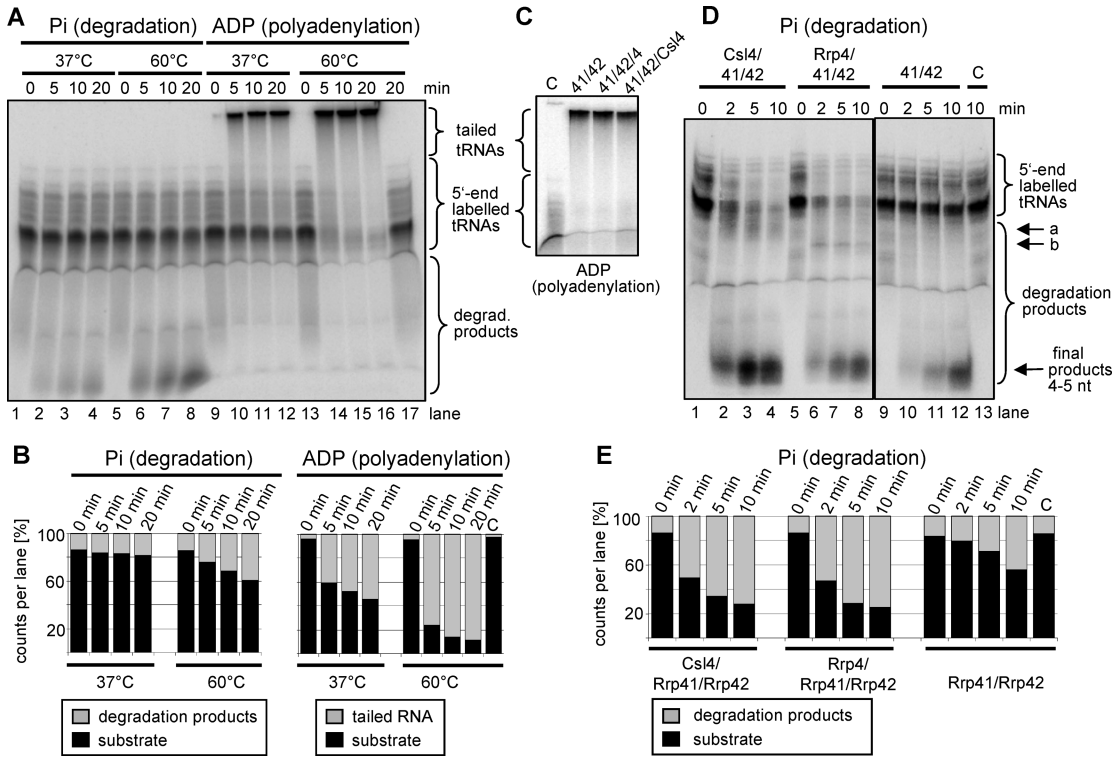

FIGURE 6: Degradation and polyadenylation of 5′-end-labeled natural tRNAs from *Sa. cerevisiae* by the hexameric core of the exosome (*Sso*Rrp41/*Sso*Rrp42) and by nine-subunit exosomes (*Sso*Rrp4/*Sso*Rrp41/*Sso*Rrp42 and *Sso*Csl4/*Sso*Rrp41/*Sso*Rrp42, respectively). (A) Phosphorimage of a 10% denaturing PAA gel showing the results of degradation and polyadenylation assays with the hexamer. The incubation temperature and the incubation time are indicated above the panel. The reaction buffer for polyadenylation assays contained 10 mM ADP instead of 10 mM P_i, which was used for degradation assays. Lane 17 was the negative control. (B) Quantitative representation of the results shown in panel A. The radioactivity per lane was set to 100%. (C) Phosphorimage of a 10% denaturing PAA gel showing the results of polyadenylation assays (2 min at 60 °C) with the complexes indicated above the panel. ADP (10 mM) was added to the reaction mixtures. (D) Phosphorimage of an 18% denaturing PAA gel showing the results of degradation assays with the complexes indicated above the panel. P_i (10 mM) was added to the reaction mixtures. The incubation time at 60 °C is indicated. Substrate and products are marked. Arrows marked with a and b denote degradation products produced by the *Sso*Csl4 and *Sso*Rrp4 exosome, respectively. (E) Quantitative representation of the results shown in panel D. The radioactivity per lane was set to 100%. The composition of the reaction buffer is given in the legend of Figure 4. Lane C was a negative control.

conditions, exosome composition, or substrate characteristics. Our data show that as expected, single-stranded poly(A) RNA is degraded better than heteropolymeric RNA which forms secondary structures and much better than structured natural tRNA, although all three types of substrates were easily polyadenylated. However, the initially used buffer is suboptimal for RNA degradation, and the hexameric ring has an intrinsic low degradation activity when compared to its polyadenylation activity.

It was possible to increase the degradation activity by omitting glycerol, increasing the level of $MgCl_2$, and lowering the pH to physiologically relevant values of 6−7 (*S. solfataricus* grows at pH 2−4, and the pH value of its cytoplasm is approximately 6.5; see ref *34*). Additionally, we found that the nine-subunit exosomes degrade single-stranded poly(A) RNA and the heteropolymeric MCS RNA much better than the hexamer. Moreover, the nine-subunit exosomes were capable of degrading tRNA, which was essentially untouched by the hexamer under the applied conditions. Since the subunits of the hexamer (*Sso*Rrp41 and *Sso*Rrp42) were co-immunoprecipitated in stoichiometric amounts with the S1 domain-containing subunits (*Sso*Rrp4 and *Sso*Csl4) from cell-free extracts (*8, 14*), the in vitro behavior of the recombinant nine-subunit exosome should be relevant for its role in the cell. In summary, our data show that the *S. solfataricus* exosome efficiently degrades synthetic and natural RNA, in accordance with its proposed role as a major 3′ to 5′ exoribonuclease in the cell.

Despite the finding that the nine-subunit exosome degrades RNA much faster than the hexameric ring, all tested substrates were polyadenylated with similar efficiency by the three complexes with different compositions. The observation that tRNAs were efficiently polyadenylated but not degraded by the hexamer shows that degradation did not succeed despite the fact that the substrates were bound and their 3′-end reached the active sites. Given that the two reactions are catalyzed by the same reaction center, and since it is known that nine-subunit exosomes bind RNA much better than the hexamer (*9, 39*), it seems that stronger binding of the substrate is more important for degradation than for polyadenylation. The relative amounts of substrate and degradation products of different sizes in assays with poly(A) RNA and different exosome complexes (for example, see Figure 1A,B and Figure 5A,B of the Supporting Information and the corresponding text in Results) suggest that the S1 domain-containing subunits of the exosome are crucial for the initiation of degradation, which seems to be performed very inefficiently by the hexamer. Once degradation starts (documented by the disappearance of the substrate band), the further decay (documented by the appearance of degradation products) seems to be performed with similar efficiency

by the three different complexes and is also influenced in a similar way by increasing glycerol and $MgCl_2$ concentrations.

The analysis of the buffer components influencing the activity of the archaeal exosome revealed that increasing glycerol and KCl concentrations inhibit the degradation and polyadenylation reactions, most probably due to weaker interactions between the enzyme and substrate. A similar influence of KCl on RNA degradation by *E. coli* RNase PH was reported previously (*37*). Changes in pH and $MgCl_2$ concentrations had different effects on the two activities of the exosome, raising the possibility that local environmental changes may influence the reaction catalyzed by the protein complex in vivo. The composition of the protein complex may also influence the direction of the reaction and/or the activity of the exosome. The *Sso*Rrp4 complex, which is prominent in vivo under standard laboratory conditions (*8, 14*), differs in its polyadenylation capability from the *Sso*Csl4 complex at different pH values. In vitro, at physiologically relevant pH values of 6−7, only the *Sso*Rrp4 exosome was capable of efficiently polyadenylating RNA. Interestingly, spermidine, which is present in the cytoplasm of *S. solfataricus* (*40*), shifted the optimum for the polyadenylation by the hexamer and the *Sso*Csl4 complex from pH 8 to 6−7, suggesting that in the cell, *Sso*Csl4 may also be involved in RNA tailing.

$MgCl_2$ was essential for the activity of the exosome, although crystal structure analyses do not point to a catalytic role of divalent cations in phosphorolysis (*3, 9, 12, 13*). Mn^{2+} is present in the exosome containing *Sso*Rrp41, *Sso*Rrp42, and *Sso*Rrp4, but it has a structural role in the protein complex (*13*). The activity of *E. coli* RNase PH was also found to be dependent on divalent cations (*37*). Attempts to replace Mg^{2+} with Mn^{2+} or Zn^{2+} at a concentration of 5 mM were not successful (not shown), pointing to a specific role for Mg^{2+}. We think that Mg^{2+} is necessary for the interaction of the hexameric ring with RNA, explaining its essential role in polyadenylation and degradation. *Sso*Rrp4 and *Sso*Csl4 do not seem to have a specific role in RNA binding, as all three exosomal complexes with different composition showed the same dependence on changes in $MgCl_2$ concentration.

In addition, Mg^{2+}, as the most abundant divalent cation in vivo, may play a regulatory role in archaeal exosome function: degradation activity increased with increasing $MgCl_2$ concentrations up to 10 mM, while the maximum of polyadenylation activity was observed at 2−6 mM $MgCl_2$. Mg^{2+} is associated with RNA and can form a complex with NDPs as well as with P_i and probably also interacts with the exosome. Such equilibria between multiple participants should exist in vivo, and therefore, they should have an influence on the activity of the exosome. Among other possibilities, we favor the following hypothesis: ADP and P_i, but not their Mg^{2+} complexes, represent the genuine exosome substrates; the complex of Mg^{2+} with ADP is more stable than the complex of Mg^{2+} ions with P_i, and changes in the concentration of Mg^{2+} determine the availability of ADP and P_i for the reactions catalyzed by the exosome. This fits well with the in vitro data with addition of $MgCl_2$ at different concentrations to the buffer (Figure 5 of the Supporting Information). Due to the association of natural RNA with hydrated Mg^{2+}, the local concentration of "free" Mg^{2+} increases when RNA is degraded and decreases when RNA is synthesized (for the ratio of associated hydrated Mg^{2+} to nucleotides, see ref *41*). During RNA degradation, NDPs are released and Mg^{2+}−NDP complexes can be formed which are more stable than the complexes of Mg^{2+} with the P_i available in the local environment. This should support further phosphorolytic RNA degradation by the exosome. In contrast, when RNA tails are synthesized by the exosome, they associate with Mg^{2+} and thus more Mg^{2+}-free NDPs should be available for further RNA polynucleotidylation. In this way, the actual direction of the reaction catalyzed by the exosome should be supported, preventing unproductive rounds of addition and release of nucleosides at the 3′-end of RNA. In agreement with the scenario described above are the results of our in vitro experiments (Figure 3), showing that different $MgCl_2$ concentrations in the reaction buffer change the ratio of ADP to P_i at which the exosome polyadenylates or degrades RNA.

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