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Crystal structure of *Escherichia coli* PNPase: Central channel residues are involved in processive RNA degradation

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ABSTRACT

Bacterial polynucleotide phosphorylase (PNPase) plays a major role in mRNA turnover by the degradation of RNA from the 3'- to 5'-ends. Here, we determined the crystal structures of the wild-type and a C-terminal KH/S1 domain-truncated mutant (ΔKH/S1) of *Escherichia coli* PNPase at resolutions of 2.6 Å and 2.8 Å, respectively. The six RNase PH domains of the trimeric PNPase assemble into a ring-like structure containing a central channel. The truncated mutant ΔKH/S1 bound and cleaved RNA less efficiently with an eightfold reduced binding affinity. Thermal melting and acid-induced trimer dissociation studies, analyzed by circular dichroism and dynamic light scattering, further showed that ΔKH/S1 formed a less stable trimer than the full-length PNPase. The crystal structure of ΔKH/S1 is more expanded, containing a slightly wider central channel than that of the wild-type PNPase, suggesting that the KH/S1 domain helps PNPase to assemble into a more compact trimer, and it regulates the channel size allosterically. Moreover, site-directed mutagenesis of several arginine residues in the channel neck regions produced defective PNPases that either bound and cleaved RNA less efficiently or generated longer cleaved oligonucleotide products, indicating that these arginines were involved in RNA binding and processive degradation. Taking these results together, we conclude that the constricted central channel and the basic-charged residues in the channel necks of PNPase play crucial roles in trapping RNA for processive exonucleolytic degradation.

Keywords: polynucleotide phosphorylase; mRNA turnover; RNase; RNA degradation; crystal structure

INTRODUCTION

Messenger RNA turnover plays a vital role in quality control of RNA biogenesis and modulation of protein expression, and as such, it regulates diverse physiological events (Song and Parker 2004; Carpousis 2007). Eukaryotic mRNAs are largely degraded by the protein complex, the exosome, from the 3′ to 5′ direction (Rajmakers et al. 2004; Houseley et al. 2006). In parallel, the bacterial polynucleotide phosphorylase (PNPase), which shares similar domain organization to the eukaryotic exosome core complex, plays a key role in the degradation of prokaryotic mRNAs (Marcaida et al. 2006).

PNPase is a 3′ to 5′ exonuclease, using phosphate to catalyze phosphorolysis of RNA and generating nucleoside diphosphates as the cleavage products (Sarkar and Fisher 2006). PNPase is evolutionarily conserved, present in almost all species from bacteria to plants and higher mammals, but not in yeasts, trypanosomes, or archaea (Baginsky et al. 2001; Leszczyniecka et al. 2002; Sarkar and Fisher 2006). Mammalian PNPase is intriguingly located in the mitochondrial intermembrane space where no RNA is present (Piovowska et al. 2003). Recent studies suggest that human PNPase has a crucial role in maintaining mitochondrial homeostasis and is induced by type I interferons, likely playing a role in antiviral defense and cellular senescence (Leszczyniecka et al. 2003; Chen et al. 2006, 2007; French et al. 2007). However, the molecular basis for the mammalian PNPase regulation of these fundamental physiological events is largely unknown. Conversely, bacterial and plant chloroplast PNPases have been studied more extensively at the molecular level. Both enzymes participate in the rapid exonucleolytic degradation of polyadenylated messenger RNAs that are generated through endonucleolytic cleavage of RNA, followed by the addition
of a poly(A) or poly(A)-rich tail (Schuster et al. 1999; Mohanty and Kushner 2000; Yehudai-Resheff et al. 2003).

*Escherichia coli* PNPase is located in the cytoplasm, and a small fraction of the enzyme is associated with Rnase E in the protein complex, the degradosome (Carpousis et al. 1994; Liou et al. 2001). The enzyme is primarily involved in the processive phosphorolytic degradation of RNA, but the reverse polymerization of ribonucleoside diphosphates has also been observed in vivo (Mohanty and Kushner 2000). Bacterial PNPases are homotrimers, each monomer containing two N-terminal RNase PH domains linking by an α-helix domain, and a C-terminal RNA-binding S1 and KH domain (see Fig. 1A). The crystal structure of *Streptomyces antibioticus* PNPase has been determined, revealing a doughnut-shaped structure, forming by six RNase PH domains in a homotrimer (Symmons et al. 2000). This overall organization of a hexameric ring structure with the RNA-binding domain associated on the top of the ring is similar to those of archaeal (Buttner et al. 2005; Lorentzen and Conti 2005; Lorentzen et al. 2005) and human exosome (Liu et al. 2006) core complexes, indicating evolutionary links in structure and function between PNPase and exosomes in RNA degradation (Symmons et al. 2002; Lin-Chao et al. 2007).

How do PNPase and exosomes bind and cleave RNA processively from the 3′-end? The current model suggests that the conserved KH and S1 domains are involved in RNA binding, since deletion of any or both of the RNA-binding domains of *E. coli* PNPase reduces its RNA binding as well as its enzyme activity (Stickney et al. 2005; Briani et al.

**FIGURE 1.** RNA-binding and digestion activities of the *E. coli* full-length PNPase and KH/S1-domain truncated mutant (ΔKH/S1). (A) Domain structures of the full-length PNPase and the truncated mutant ΔKH/S1. (B) RNA-binding activity of full-length PNPase and ΔKH/S1 were analyzed by gel shift assays. The 20-mer (left panel) and 8-mer (right panel) single-stranded RNA substrates (0.1 pmol) were incubated with PNPase in various concentrations from 4 to 200 nM. A control reaction without any PNPase added is shown in lane C. The ΔKH/S1 mutant bound RNA less efficiently than full-length PNPase. (C) RNase activities of full-length PNPase and ΔKH/S1 mutant were assayed by incubation of enzymes, respectively, with 8-mer and 20-mer single-stranded RNAs in time course experiments under the conditions described in Materials and Methods. The left panel shows that ΔKH/S1 had lower activities in cleaving 20-mer ssRNAs compared to the full-length PNPase. The right panel shows that ΔKH/S1 cannot degrade shorter 8-mer ssRNAs. (D) The dissociation constants between PNPase and the 20-mer ssRNA were estimated by gel shift assays.
substrates. The EMSA (Fig. 1B) showed that the full-length using a 20-mer and an 8-mer single-stranded RNA as binding activities by electrophoresis mobility shift assays, D

17–734) and the KH/S1 domain-truncated mutant (Fig. 1A, mutant. We purified full-length PNPase (Fig. 1A, residues efficiencies of wild-type PNPase and KH/S1 domain-truncated protein secondary structures, not quaternary structures, we measured the thermal melting points of full-length PNPase and ΔKH/S1 mutant by circular dichroism (CD) (Fig. 2A). The estimated melting point of full-length PNPase was 55°C, and that of ΔKH/S1 was 45°C, 10 degrees lower than the wild-type protein. This result showed that ΔKH/S1 was less thermal stable than the wild-type PNPase.

Since CD measurements reflect the thermal melting of protein secondary structures, not quaternary structures, we further analyzed the particle size of PNPase during thermal denaturation and acid-induced dissociation by dynamic light scattering (DLS). Thermal denaturation typically leads to an increase in protein particle size due to the aggregation of the unfolded proteins (Santiago et al. 2008). We

RESULTS
The KH/S1 truncated PNPase binds and cleaves RNA less efficiently
E. coli PNPase contains a C-terminal KH and an S1 domain, which have been identified in a number of nucleic acid-binding proteins (Theobald et al. 2003). Previous studies showed that the KH and S1 domains in PNPase are involved in RNA binding (Stickney et al. 2005; Briani et al. 2007; Matus-Ortega et al. 2007). To further study the interactions between PNPase and RNA, we quantitatively measured the RNA binding affinities and RNA cleavage efficiencies of wild-type PNPase and KH/S1 domain-truncated mutant. We purified full-length PNPase (Fig. 1A, residues 17–734) and the KH/S1 domain-truncated mutant (Fig. 1A, ΔKH/S1, residues 17–570), and compared their RNA binding activities by electrophoresis mobility shift assays, using a 20-mer and an 8-mer single-stranded RNA as substrates. The EMSA (Fig. 1B) showed that the full-length PNPase bound 20-mer and 8-mer RNA at concentrations >4 nM, whereas the truncated mutant ΔKH/S1 bound 20-mer and 8-mer RNA at concentrations >∼20 nM. A further quantitative gel shift study showed that wild-type PNPase bound the 20-mer RNA with a K_d of 15.6 ± 1.8 nM, whereas ΔKH/S1 bound the 20-mer RNA with an eightfold reduced affinity with a K_d of 132.3 ± 12.1 nM. This result is consistent with earlier studies (Stickney et al. 2005; Briani et al. 2007), suggesting that PNPase without S1/KH domains binds RNA less efficiently with an approximately one-order reduced binding affinity than full-length PNPase, and suggesting that the KH/S1 domain in PNPase is involved in RNA binding.

We then tested the exoribonucleolytic activity of full-length PNPase and ΔKH/S1 mutant in time course activity assays. The full-length PNPase digested both 20-mer and 8-mer RNA into small oligonucleotides, as shown in Figure 1C. The cleavage efficiencies of PNPase are indicated at the bottom of the gel, showing that the RNA substrates were cleaved processively with the cleavage percentage of 87%–94% in the reaction time of 1–10 min. However, ΔKH/S1 not only had less enzyme activity when cleaving 20-mer RNA, it also generated longer degradation products of ∼12-mer RNAs. The cleavage efficiencies were reduced to 32%–81% in the reaction time of 1–10 min. A 20-mer RNA and 8-mer RNA with different sequences were also analyzed for binding and cleavage assays and similar results were obtained (data not shown), verifying that all the altered properties of the truncated mutant were not sequence specific. Moreover, ΔKH/S1 could not degrade the shorter 8-mer RNA. Since ΔKH/S1 is able to bind 8-mer RNA (as shown by EMSA), it is intriguing why it cannot cleave 8-mer RNA. These results imply that the KH/S1 domain is involved not only in RNA binding, but also in modulation of enzyme activity in an unknown way.

The KH/S1 domain is involved in trimer formation
An earlier report showed that the S1 domain from PNPase was able to induce the trimerization of a R Nas e II-PNPase hybrid protein (Amblar et al. 2007). To test if KH/S1 domain in PNPase is involved in homotrimer formation, we measured the thermal melting points of full-length PNPase and ΔKH/S1 mutant by circular dichroism (CD) (Fig. 2A). The estimated melting point of full-length PNPase was 55°C, and that of ΔKH/S1 was 45°C, 10 degrees lower than the wild-type protein. This result showed that ΔKH/S1 was less thermal stable than the wild-type PNPase.

Since CD measurements reflect the thermal melting of protein secondary structures, not quaternary structures, we further analyzed the particle size of PNPase during thermal denaturation and acid-induced dissociation by dynamic light scattering (DLS). Thermal denaturation typically leads to an increase in protein particle size due to the aggregation of the unfolded proteins (Santiago et al. 2008). We
observed similar phenomena that wild-type PNPase had a homogeneous trimeric particle size at the temperature of $20^\circ C$ and $40^\circ C$, and it was partially denatured with an additional peak of large aggregates at $60^\circ C$ (see Fig. 2B). On the other hand, $\Delta KH/S1$ had a homogeneous trimeric size only at $20^\circ C$ and it started to melt at lower temperatures of $40^\circ C$ and $60^\circ C$, indicating that $\Delta KH/S1$ was a less thermal-stable trimer than the wild-type PNPase.

The oligomeric proteins can be dissociated into monomers by acidic or basic conditions. We induced the trimer dissociation by decreasing the pH of protein solutions from 7.5 to 3.5 at $20^\circ C$ (see Fig. 2C). The wild-type PNPase was a homotrimer up to pH 4.5, and it dissociated into monomers at pH 3.5, whereas $\Delta KH/S1$ was a homotrimer at pH 7.5 and 6.5, and it dissociated into monomers at pH 5.5. This result showed that the truncated PNPase, without the KH/S1 domain, was melted at lower temperatures and dissociated into monomers in less acidic solutions. Therefore, we conclude that the KH and S1 domains contribute to the formation of a more stable trimeric PNPase.

Crystal structures of full-length and $\Delta KH/S1$ PNPase

To find out why the truncated mutant $\Delta KH/S1$ had different biochemical properties, we crystallized the two proteins for structural determination. The full-length PNPase crystallized in the cubic space group F4$_1$32, diffracting X-rays to a resolution of 2.6 Å, whereas $\Delta KH/S1$ mutant crystallized in the rhombohedral space group R32, diffracting X-rays to a resolution of 2.8 Å. Both structures were solved by molecular replacement using the crystal structure of *Streptomyces antibioticus* PNPase (PDB accession code...
1E3P) as the searching model. The statistics for X-ray diffraction and structural refinement are listed in Table 1.

The final model of the full-length PNPase crystal structure contained two RNase PH domains and one α-helical linker domain from residues 17 to 565 (see Fig. 3). The C-terminal KH and S1 domains (Fig. 1A, residues 566–734) were not visible, containing only broken densities in the electron density maps. These KH and S1 domains were likely disordered as a whole in the crystal structure since they were not visible, containing only broken densities in the electron density maps. These KH and S1 domains were not visible, containing only broken densities in the electron density maps.

Superposition of E. coli full-length PNPase to that of Streptomyces antibioticus gave an RMSD of 1.6 Å over 443 Cα atoms, suggesting that the two bacterial structures are highly similar, whereas superposition of ΔKH/S1 to that of Streptomyces antibioticus gave a slightly higher RMSD of 1.83 Å over 384 Cα atoms.

As expected, the homotrimer of PNPase forms a ring-like structure containing a central channel. The halved trimeric PNPase, displayed in Figure 3D, shows that the central channel contains two constricted necks with three arginine residues located in the neck regions: Arg102 and Arg103 in the upper neck closer to the channel entrance, and Arg106 in the lower neck, closer to the active site located in the second RNase PH domain. Both of the RNase PH domains, PH1 and PH2, contribute to the channel surface inside of the PNPase (surface displayed respectively in blue and yellow in Fig. 3D).

The overall structure of ΔKH/S1 was similar to that of the full-length PNPase. Superposition of only one of the monomers (Fig. 4A, molecule a) of the full-length and ΔKH/S1 PNPase gave an average RMSD of 1.1 Å for 417 Cα atoms. Thus, these results support the hypothesis that the KH/S1 domain in PNPase is involved in trimeric assembly. Without this KH/S1 domain, the PNPase trimer is more loosely packed, bearing a larger central channel.

### TABLE 1. X-ray data collection and refinement statistics for the full-length E. coli PNPase and the KH/S1 domain-truncated PNPase mutant (ΔKH/S1)

<table>
<thead>
<tr>
<th>Data collection statistics</th>
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<th>ΔKH/S1</th>
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<tbody>
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<td>Space group</td>
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<td>R32</td>
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<tr>
<td>Cell dimensions (Å)</td>
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<td>a = 160.08</td>
</tr>
<tr>
<td></td>
<td>b = 270.12</td>
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<td></td>
<td>c = 270.12</td>
<td>c = 153.15</td>
</tr>
<tr>
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<td>1.00</td>
</tr>
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<td>50.0–2.8 (2.9–2.8)</td>
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<td>Unique reflections</td>
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<td>19.7 (13.4)</td>
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<td>Rmerge (%)</td>
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<td>5.0 (15.9)</td>
</tr>
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<td>Refinement statistics</td>
<td></td>
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<tr>
<td>Resolution range (Å)</td>
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<td>50.0–2.8</td>
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<td>Rwork/Rfree (%)</td>
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<td>27.6/-29.4</td>
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<tr>
<td>Number of atoms (protein/water)</td>
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<tr>
<td>Average B-factor (Å°)</td>
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<td>61.8</td>
</tr>
<tr>
<td>RMS deviations (bond length [Å]/ bond angle [degree])</td>
<td>0.007/1.422</td>
<td>0.017/2.85</td>
</tr>
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</table>

*Highest resolution shell is shown in parentheses.

Arginine residues in the channel neck regions are involved in RNA binding and processing

Previously published crystal structures of an archaeal exosome in complex with RNA substrates show a ribonucleotide bound at the narrowest constriction of the central channel (Lorentzen et al. 2007; Navarro et al. 2008). To find out if RNA is also bound at the neck regions in PNPase, we mutated the two arginine residues located at the upper neck (R102 and R103) closer to the channel entrance, and one arginine located at the lower neck (R106) closer to the active site, to generate the double-mutant R102A/R103A and a single-point mutant R106A. R102A/R103A had decreased binding activity to a 20-mer or an 8-mer...
ssRNA, compared to the wild-type PNPase, as shown in Figure 5A. The dissociation constant between R102A/R103A and the 20-mer RNA was 378.2 ± 32.1 nM, about 24-fold reduced binding affinity compared to the wild-type PNPase. On the other hand, R106A bound the 20-mer RNA substrate as efficiently as wild-type PNPase with a $K_d$ of 5.2 ± 0.6 nM, even though it bound the 8-mer RNA less efficiently. This indicates that the arginines located in the upper neck are involved in capturing RNA substrates, whereas the arginine located in the lower neck is not a determinant factor in the first step of RNA binding.

We then compared the RNase activities of wild-type and mutated enzymes. R102A/R103A produced similar cleaved products when it digested 20-mer and 8-mer ssRNA substrates, although less efficiently than wild-type PNPase (Fig. 5B). Conversely, R106A was almost as efficient as wild-type PNPase in digesting 20-mer ssRNA, because about 64% of RNA substrates were degraded after 1-min incubations (compared to 87% for the wild-type PNPase). However, R106A could not degrade 20-mer RNA to smaller products, and instead yielded a set of stalled intermediates even after 5-min or 10-min incubations. Moreover, R106A could not degrade shorter 8-mer ssRNA substrates at all. These biochemical properties of R106A were similar to those of the DKh/S1 mutant, which cleaved only longer RNA and produced longer products of digestion.

**DISCUSSION**

**The KH/S1 domain helps PNPase to form a more stable compact trimer**

Our biochemical, circular dichroism, dynamic light scattering, and structural results consistently suggest that the
KH/S1 domains are involved not only in RNA binding but also in the formation of a compact trimer with a more constricted central channel. Is it a general phenomenon that the S1 RNA-binding subunit in exosomes also modulates complex formation and channel size? Extensive structural studies have been carried out on archaeal exosome core enzymes, which contain three copies of Rrp41 and Rrp42, each bearing a RNase PH domain, to form a hexameric ring-like structure. One of the S1 homologues, Rrp4 or Csl4, is bound on the top of the ring to form a trimeric S1 pore (Lorentzen et al. 2007). The crystal structures of *Sulfolobus solfataricus* exosome core enzyme Rrp41/Rrp42 in the presence or absence of the RNA-binding subunit of Rrp4 have been solved (Lorentzen et al. 2005; Lorentzen et al. 2007). Interestingly, the central channel of trimeric Rrp41/Rrp42, shown in Figure 6A, appears to be wider than that of the Rrp4/Rrp41/Rrp42 complex. This suggests that Rrp4 may also contribute to complex formation, where it likely helps the nine-subunit exosome to assemble into a more compact structure. Moreover, the surface area buried in the interface of Rrp41 and Rrp42 between two heterodimers are 1210 Å² (for each monomer) in the presence of Rrp4, and the buried surface is reduced to 1080 Å² in the absence of Rrp4, supporting that KH and S1 domains in Rrp4 contribute to the formation of a more compact exosome.

The crystal structures of two isoforms of *Archaeoglobus fulgidus* exosome, Rrp4/Rrp41/Rrp42 and Csl4/Rrp41/Rrp42, have been reported (Buttner et al. 2005). The channels of these two complexes are different in shape and size (see Fig. 6B). The channel in Cal4/Rrp41/Rrp42 is more curved and slightly larger than that in Rrp4/Rrp41/Rrp42. This result indicates that the RNA-binding subunit located on the top of the hexameric ring may modulate channel shape allosterically. Our *E. coli* ΔKH/S1 PNPase mutant does not bind RNA as well as full-length PNPase, likely because the KH/S1 domain pair is responsible for direct interaction with RNA. Additionally, without the KH/S1 domains, the size of the channel is also changed accordingly. A wider channel is likely to be less efficient at trapping RNA for continuous degradation, and therefore leads to a defect enzyme in processive degradation, i.e., it cannot effectively cleave shorter RNAs and produces longer cleaved products.

**A constricted central channel plays a crucial role in RNA binding and processing**

How does the channel grasp single-stranded RNA substrates? The structure of the archaeal exosome in complex with RNA demonstrated that a single-stranded RNA is likely threaded through the S1 pore and bound at a constricted neck, where a conserved basic arginine residue (Arg65 in Rrp41) is responsible for the interactions with RNA (Lorentzen et al. 2007). In *E. coli* PNPase, two necks, upper and lower, are identified in the crystal structure (marked by arrows in Figs. 3, 4). These necks are formed by arginine residues in the first RNase PH domain, Arg102/Arg103 constituting the upper neck, and Arg106 constituting the lower neck. Mutation of Arg102/Arg103 in the
upper neck produced a mutant less efficient in RNA binding and cleavage. The decreased RNase activity in cleaving a 20-mer RNA is a reflection of the decreased RNA-binding activity, since both activities reduced about 10- to 20-fold. This result suggests that the constricted neck formed by basic residues close to the channel entrance is involved in capturing RNA. Both the basic charged residues and the size of the channel contribute to the binding of RNA.

The mutation of Arg106 located in the lower neck produced an interesting defective mutant in R106A, which was as efficient as wild-type PNPase, although it could not degrade a shorter 8-mer ssRNA and the degradation reaction stalled whenever the RNA was trimmed to z nt. This phenotype is similar to that of KH/S1 truncated PNPase. Based on the crystal structure of KH/S1, we know that the truncated mutant has a wider central channel. Mutation of Arg106 to alanine likely produced similar effects since removal of the three copies of Arg106 side chain in the channel would lead to a bigger channel at the lower region as well. A wider neck must be disadvantageous for RNA binding, as RNA cannot be properly bound here to allow a continuous feed of substrate into active sites, thus leading to longer cleaved RNA products. We, therefore, suggest that the lower neck close to the active site is involved in processive RNA degradation.

A further structural comparison to the Streptomyces antibioticus PNPase shows that the S. antibioticus PNPase also has two constricted necks in its RNA-binding channel, one closer to channel entrance, one closer to the active site, similar to that of E. coli PNPase (see Fig. 6C). On the other hand, the inactive human exosome has a channel without obvious constricted necks (see Fig. 6C). This observation supports our suggestions that necks are important in trapping RNA substrates for degradations, and therefore only the bacterial PNPases and archaeal exosomes, with high RNase activities, have obvious constricted neck regions. Moreover, the three arginine residues located in the neck regions are highly conserved in PNPase (see Fig. 3), suggesting that these necks are also conserved features in PNPase. Together with the structural data from archaeal exosomes, we conclude that the KH/S1 domains and the upper neck in the channel recruit RNA into the channel, and the lower neck keeps hold of the RNA as it guides it into the PNPase active site for processive degradation.

Constrictions of the appropriate size at the necks in the central channel of exosomes and PNPases play a crucial role in RNA binding and processive degradation.

MATERIALS AND METHODS

Cloning, protein expression, and purification

The genes of the full-length PNPase (residues 17–734) and truncated ΔKH/S1 mutant (residues 17–570) were amplified by PCR and subcloned respectively into Ndel/Xhol sites of expression vectors pET-22b and pET-28c (Novagen) to generate N-terminal
His-tagged constructs. All PNPase point mutants were generated by Quickchange site-directed mutagenesis kits (Invitrogen). All expression plasmids were transformed into the BL21 (DE3) strains by Quickchange site-directed mutagenesis kits (Invitrogen). All PNPase point mutants were generated as His-tagged constructs. All PNPase point mutants were generated as His-tagged constructs.

**RNA-binding and RNase activity assays**

Electrophoresis mobility shift assays (EMSA) were preformed using a single-stranded 20-mer RNA: 5′-ACUGGACAAAUA CUCCGAGG-3′ and an 8-mer RNA: 5′-AAAAAAA-3′, as substrates. The RNA substrates were labeled first at their 5′ end with [γ-32P]ATP by T4 polynucleotide kinase, and then purified by a Microspin G-25 column (GE Healthcare) to remove the nonincorporated nucleotides. The RNA substrate (0.1 pmol) was then incubated with different concentrations of PNPase (4 to 200 nM) in a buffer containing 20 mM Tris-HCl pH 7.6, 100 mM KCl, 2 mM DTT, 10 mM ZnCl2 at 37°C for 10 min. The ZnCl2 was added to ensure that PNPase can only bind but cannot cleave RNA substrates since zinc ions inhibited the enzyme activity. After incubation, the reaction mixture was applied to 20% TBE gels, which were exposed to the phosphorimaging plate (Fujifilm) for autoradiographic visualization.

For RNase activity assays, 0.1 pmol of single-stranded RNA substrates were incubated with wild-type or mutated PNPase (400 nM) in a reaction buffer containing 20 mM Tris-HCl pH 7.6, 100 mM KCl, 2 mM DTT, 1 mM MgCl2, and 5 mM KH2PO4 at 37°C. The reaction was stopped at the time point indicated in the figures by adding TBE-Urea sample buffer (Bio-Red). Reaction products were resolved in 15% polyacrylamide/7 M urea gels, which were exposed to the phosphorimaging plate (Fujifilm) and analyzed by the imaging system FLA-5000 (Fujifilm).

**Circular dichroism (CD)**

The thermal denaturing melting points of PNPase were measured three times by a circular dichroism spectrometer AVIV CD400. The CD spectra were scanned from 25°C to 85°C at a wavelength of 223 nm and the melting point was estimated by AVIV program. The protein concentration was 0.1 mg/mL in a buffer containing 25 mM sodium phosphate (pH 7.2).

**Dynamics light scattering (DLS)**

DLS measurements were carried out on a Dyna-Pro 99 MS800 instrument (Protein Solutions). The wild-type PNPase and ΔKH/S1 mutant (0.5 mg/mL) in a buffer of 50 mM potassium phosphate (pH 7.5) and 20 mM NaCl were filtered with a 0.1-μm Anodisk filter, and placed in a 12-μL cuvette (b = 1.5 mm). The protein samples were incubated for 5 min in different temperatures (20°C, 40°C, and 60°C) before data acquisition over an acquired time of 15 min.

For the analysis of the acid-induced trimer dissociation, the PNPase samples in buffers of different pH, ranging from 3.5 to 7.5 were measured at 20°C (pH 6.5 and 7.5:50 mM potassium phosphate buffer; pH 5.5, 4.5, and 3.5:50 mM glycine-HCl buffer). The size distribution plots, the x axis showing a distribution of estimated particle radius (nm) and the y axis showing the relative intensity of the scattered light (% of mass), were analyzed and prepared with the software Dynamics V5.26.60 (Protein Solutions).

**Crystallization and data collection**

Both full-length and ΔKH/S1 mutant were concentrated to 10 mg/mL in a buffer of 10 mM NaCl and 20 mM Tris-HCl at pH 7.0. Crystals of full-length PNPase were grown by hanging drop vapor-diffusion method at room temperature, by mixing 1 μL of protein solution with 1 μL of reservoir solution containing 15% w/v PEG 4000, 2 M MgCl2, and 0.1 M Tris-HCl at pH 8.5. Crystals of ΔKH/S1 mutant were grown by the same method using a reservoir containing 1.5 M ammonium sulfate and 0.1 M Tris-HCl at pH 8.5. Diffraction data were collected at −150°C at beamline 13C1.
of the NSRRC in Hsinchu, Taiwan, and were processed and scaled by HKL2000 (Otwinowski and Minor 1997). All diffraction statistics are listed in Table 1.

Structure determination and refinement

The full-length PNPase crystallized in the F4_32 cubic space group, whereas the ΔKH/S1 mutant crystallized in the rhombohedral space group of R32, with one molecule per asymmetric unit in both crystal systems. The structures of full-length PNPase and ΔKH/S1 were solved by molecular replacement by EPMR, using the crystal structure of Streptomyces antibioticus PNPase (PDB accession code: 1E3P) as the searching model. The structure model was subjected to manual rebuilding with WinCoot and then refined with the program CNS. Structural coordinates and diffraction structure factors have been deposited in the RCSB Protein Data Bank with PDB ID codes of 3CDI for full-length PNPase and 3CDJ for the ΔKH/S1 mutant.

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