Probing the Functional Importance of the Hexameric Ring Structure of RNase PH*

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RNase PH is a phosphate-dependent exoribonuclease that catalyzes the removal of nucleotides at the 3’ end of the tRNA precursor, leading to the release of nucleoside diphosphate, and generates the CCA end during the maturation process. The 1.9-Å crystal structures of the apo and the phosphate-bound forms of RNase PH from Pseudomonas aeruginosa reveal a monomeric RNase PH with an αβ-fold tightly associated into a hexameric ring structure in the form of a trimer of dimers. A five ion pair network, Glu-63-Arg-74-Asp-116-Arg-77-Asp-118 and an ion-pair Glu-26-Arg-69 that are positioned symmetrically in the trimerization interface play critical roles in the formation of a hexameric ring. Single or double mutations of Arg-69, Arg-74, or Arg-77 in these ion pairs leads to the dissociation of the RNase PH hexamer into dimers without perturbing the overall monomeric structure. The dissociated RNase PH dimer completely lost its binding affinity and catalytic activity against a precursor tRNA. Our structural and mutational analyses of RNase PH demonstrate that the hexameric ring formation is a critical feature for the function of members of the RNase PH family.

The maturation process of the longer precursor of RNA into a functional form is essential for cell growth and viability (1). Multiple endoribonucleases and exonucleases (exoRNases) are involved in this process to remove additional ribonucleotides from both ends of the RNA precursor (2–4). For tRNA, a series of processing reactions produces the double-stranded terminus at the 5’ end and the CCA terminus at the 3’ end for aminoacylation and protein synthesis (3–5).

In bacterial tRNA-processing reactions, the removal of the 5’ extension sequence is a single step involving RNase P, a conserved endoribonuclease in all organisms (6). However, the trimming of the 3’ end requires several endo- and exoRNases; initially endoribonucleolytic cleavage occurs downstream of the CCA sequence (3, 7), and then a series of exoRNases, including RNase II, BN, T, D, and PH, removes the remaining ribonucleotides (8). All exoRNases, RNase PH and RNase T are known to be the most effective (9).

RNase PH is unique among all the exoRNases that participate in the tRNA maturation process in that it catalyzes the degradation of the 3’ end in a phosphate-dependent manner (10, 11). It also shows synthetic activity; that is, the addition of ribonucleotides to the 3’ end of RNA with nucleoside diphosphate and inorganic phosphate (12). In addition to the tRNA precursor, RNase PH can also cleave off the 3’ end of other small RNAs, including M1, 6 S, and 4.5 S RNA (13). Although the sequences of these RNAs are significantly different, their structures are homologous to that of tRNA, and thus, it is likely that RNase PH recognizes and digests the substrate in a structure-specific manner rather than a sequence-specific manner (13).

Another known phosphorolytic exoRNase, polynucleotide phosphorylase (PNPase), can also cleave the 3’ end of a precursor tRNA (10). However, unlike RNase PH, PNPase shows an almost 100-fold lower relative activity on precursor tRNA compared with the nuclease activity on poly(A) (10), implying that the primary role of PNPase is in mRNA degradation (14). Recently, the crystal structure of Streptomyces antibioticus PNPase (Sa PNPase) has been determined (15). It shows that the PNPase forms a trimeric ring structure, where the central core of each monomer contains two repeats of a domain that we will term the “RNase PH-like domain,” since each repeated domain shares about 20% of its sequence identity with RNase PH from Escherichia coli (Ec RNase PH) (Fig. 1a; Refs. 15–17). In PNPase, the putative active site is located in the second RNase PH-like domain, where the side chains of residues His, Thr, and Ser are liganded to the tungstate ion (15, 17). Three additional domains that are homologous to other RNA binding domains are present on each face of the PNPase ring (15).

Despite the structural and mutational analysis of PNPase (15, 17) and the extensive biochemical studies of RNase PH (7–10), little is known about (i) whether the hexameric (or trimeric) ring structure formation is a conserved feature in the phosphorolytic exoRNase family, (ii) the functional significance of the ring structure in members of this family, and (iii) the binding mode and the catalytic mechanism of RNase PH against the substrate RNAs. During the preparation of this manuscript, the structure of RNase PH from hyperthermophilic Archea Aquifex aeolicus (Az RNase PH) has been reported (18). Although this study properly addresses several features of Az RNase PH, including a phosphate-binding site, it is unclear from this study if the formation of the hexameric ring structure is significant for the exoRNase function of RNase PH. It has been reported that Ec RNase PH exists in different oligomeric forms including a dimer (11), and thus, it is unknown whether the hexameric ring formation is required for binding to tRNA or for exoRNase activity.

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FIG. 1. The overall structure of Pa RNase PH. a, the trimeric ring structure of Sa PNPase. Three colors in light and dark pairs have been used, where lighter colors show the RNase PH-like domains with greater structural resemblance to the RNase PH monomer. The orientations of each of the RNase PH-like domains within the central core of PNPase are identical to those in c. b, additional domains within Sa PNPase are omitted for clear comparison with the structure of Pa RNase PH in c. A monomer structure of Pa RNase PH is shown in a ribbon representation. A phosphate ion is shown in a ball-and-stick model (gray, oxygen; green, phosphate). c, stereodiagram showing the hexameric ring structure of Pa RNase PH. Each monomer in the dimer is colored in cyan and yellow. The dimerization and trimerization interfaces are indicated by open circles and open triangles, respectively. The intersubunit β sheets have been highlighted in green and magenta. A phosphate ion is shown in a Corey-Pauling-Koltun model. The N-terminal and C-terminal ends of each monomer are labeled. d, bar graphs showing the buried surface area of each residue in the dimerization interface (left) and in the trimerization interface (right).
In this study, we determined the crystal structures of RNase PH from *Pseudomonas aeruginosa* (Pa RNase PH) in apo form and in phosphate-bound form at 1.9- and 2.0-Å resolution, respectively. The crystal structures revealed a conserved ion-pair network and an ion-pair at the interface that appear to be critical for the formation of a hexamer. Based on this information, we carried out mutagenesis analyses on the arginine residues forming these ion-pairs and demonstrated that the hexameric ring formation is essential for the catalytic activity on the 3' end of tRNA as well as the binding with precursor tRNA.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification**—DNA encoding Pa RNase PH was amplified by PCR, inserted into a pQE-30 vector using BamH1 and HindIII sites, and expressed in *E. coli* SG13009. The N-terminal His-tagged proteins were purified as suggested (Qiagen). Briefly, cells were harvested by centrifugation and lysed by sonication in 50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1 mM 2-mercaptoethanol. The lysate was clarified by centrifugation, and the protein was purified by a nickel column. The eluted proteins were further purified using Mono Q by NaCl gradient (0–500 mM). Finally, the gel filtration column was equilibrated with a buffer containing 5 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5 mM DTT. The proteins were finally concentrated to 20 mg/ml by ultrafiltration and stored at −70 °C until use.

For the selenomethionine-substituted Pa RNase PH, B834(DE3) was used as a methionine auxotroph strain. Cells were cultured at 37 °C in selenomethionine minimal media. The protein was purified by the same method of native protein purification. For gel filtration analyses, wild-type and mutant proteins at a concentration of 2 mg/ml in a buffer (20 mM potassium phosphate, pH 7.4, 150 mM NaCl, 5 mM DTT) were loaded into a Superdex 200 column (Amersham Biosciences).

**Crystallization and Data Collection**—The Pa RNase PH crystals were grown by the hanging-drop vapor diffusion method. Briefly, the protein (20 mg/ml) in 5 mM Tris-HCl, pH 8.0, with 150 mM NaCl and 5 mM DTT was mixed with reservoir solution (1.2 M ammonium sulfate and 0.1 M NaCl in 0.2 M CHES buffer, pH 9.5) at an equal ratio. Four microliters of this mixture was equilibrated with 500 μl of reservoir solution at 18 °C. The resulting crystals also belong to the space group R32 with identical cell dimensions to those grown from ammonium sulfate. Data were processed with the programs DENZO and SCALEPACK (19).

**Table I**

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1.0 mM ammonium phosphate, and 200 mM NaCl. Four microliters of this mixture was equilibrated with 500 μl of reservoir solution at 18 °C. The resulting crystals were grown by the hanging-drop vapor diffusion method. Briefly, the protein (20 mg/ml) in 5 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 10 mM NaH2PO4, 150 mM NaCl, and 5 mM DTT was loaded into a Superdex 200 column (Amersham Biosciences).

**Substrate Binding Assay**—For the substrate binding assay of Pa RNase PH, a tRNA3′ was prepared with two additional nucleotides in the 3′ end was prepared (tRNA3′-CCA-N) as a methionine auxotroph strain. Cells were cultured at 37 °C from a crystal flash-frozen in crystallization buffer containing 30% sucrose using the B6 beamline in the Pohang Accelerator Laboratory. The crystals were also grown by mixing an equal volume of protein in 5 mM Tris-HCl, pH 8.0, with 150 mM NaCl and 5 mM DTT and reservoir solution containing 100 mM citrate, pH 5.5.
containing 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl2, 150 mM NaCl, and 300 nM of tRNAVal-CCA-N2. After incubation for 15 min at 18 °C, the samples were resolved by 5% non-denaturing polyacrylamide gel and visualized with ethidium bromide.

RESULTS

Overall Structure of Pa RNase PH—The monomer of Pa RNase PH folds into a single domain of β-sheet comprised of nine β strands and six α helices (Fig. 1b). Two central helices, H2 and H4, are sandwiched between two β sheets, composed of S1 to S5 and S6 to S9. One long helix, H6, is packed against a second sheet (S6 to S9) and exposed to the surface, forming a ββαβ structure (Fig. 1b). Fig. 1c shows the hexameric ring structure of Pa RNase PH, where each monomer is associated into a trimer of dimers by crystallographic symmetry operations. The gel filtration studies show that wild-type Pa RNase PH forms a hexamer in solution, consistent with the findings of our crystallographic analysis (see Fig. 5a).

In the center of the hexameric ring a small channel with a diameter of 6 Å is formed by the H1 and the L5 loop (Fig. 1c). The inner surface of the ring is formed by the first sheet (S1 to S5) and the H2 helix. In the outer surface, four strands from the second sheet (S6 to S9) are connected to those from the adjacent monomer to form an eight-stranded β sheet, and the C-terminal helix H6 is packed with another H6 in an antiparallel manner.

In the phosphate-bound form of the crystal, a phosphate ion is located in the cleft formed by portions of the helices H2, H4, and H5, strands S6, S8, and S9, an L4 loop from the monomer, and the H2, S4, and L3 loop from an adjacent monomer, indicating that a phosphorolytic reaction might occur at this region of Pa RNase PH (Fig. 1, b and c).

Fig. 1c illustrates a hexameric ring of Pa RNase PH that is viewed along the crystallographic 3-fold axis. In the hexameric ring a monomer is rotated by the crystallographic 2-fold axis that is perpendicular to the 3-fold axis to generate a Pa RNase PH dimer. This orients a phosphate-binding site in the next monomer to face the opposite direction. Consequently, each face of the hexameric ring contains three phosphate-binding sites.

For convenience we will term the interfaces created upon formation of a dimer and a trimer as the “dimerization interface” and “trimerization interface,” respectively, throughout the text. The dimerization interface consists of the helices H2 and H6, and a strand S9 from each monomer (Fig. 1, c and d) and the trimerization interface is formed from the L4 loop and the β sheet consisting of S2 to S5 in the adjacent monomer (Fig. 1, c and d).

Phosphate-binding Site—The overall structure of the apoPa RNase PH is essentially identical to that of the phosphate-bound form of Pa RNase PH, with a root mean square (r.m.s.) deviation of 0.2 Å for 239 Ca atoms. In the crystal structure of the phosphate-bound form of Pa RNase PH, a strong density over 7 σ was observed in the deep cleft formed in the dimerization interface, and we have modeled a phosphate ion into this density. In an apo form of the structure, a sulfate ion is located in this position. The cleft in Pa RNase PH is narrow, with a diameter of 8 Å, and a large part of this cleft is formed from the three helices (H2, H4, and H5), three strands (S6, S8, and S9), and a loop (L4) from one monomer together with a helix (H2), a strand (S4), and a loop (L3) from an adjacent monomer (Fig. 1c and Fig. 2). In addition, residues from a strand (S3) and a loop (L3) of another dimer are on the top of the cleft, participating in the formation of this cleft (Fig. 1c and Fig. 2).

Within this deep and narrow cleft a phosphate ion resides on one side of the monomer and is liganded to four residues from the monomer and two water molecules; the side chains of Arg-87 and Arg-127 form ion pairs with the oxygen atoms of the phosphate ion (Fig. 3). The hydroxyl group of Thr-126, a conserved residue in both Pa RNase PH and PNPase, makes an H-bond to the phosphate ion. The main-chain amide group of Gly-125 also makes an H-bond to the phosphate ion. The main-chain amide group of Gly-125 also makes an H-bond to the phosphate ion.

mer to face one direction and directs another phosphate-binding site in the next monomer to face the opposite direction. Consequently, each face of the hexameric ring contains three phosphate-binding sites.

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phosphate ion. Two water molecules form H-bonds with the oxygen atom of the phosphate ion. The structure of the phosphate-binding site of the apo Pa RNase PH is virtually identical to that of the phosphate-liganded form of the Pa RNase PH; a phosphate ion has shifted by 0.2 Å compared with the position of the sulfate ion in the apo Pa RNase PH, and a root mean square deviation between the residues surrounding the phosphate ion in two structures is less than 0.2 Å.

Oligomerization Interface—The dimerization interface forms through the packing of portions of H6, the backbone of the S9, and an L8 loop from one monomer and the equivalent regions from another monomer (Fig. 1c). Interactions between residues from the helix H2 in one monomer and from the H2 in the adjacent monomer also contribute to this interface, which has a buried surface area of 2418 Å² (Fig. 1d). Among the buried groups, 20% are polar groups, 23% are charged groups, and 57% are non-polar groups. The interactions between two monomers are mediated by a compact hydrophobic core of about 10 side chains in the N-terminal half of two H6 helices from each monomer; Val-190, Met-192, Phe-198, Val-201, and Leu-219 from one monomer and Pro-209, Phe-210, and Leu-215 from another monomer are clustered together to form this hydrophobic core. In addition to van der Waals contacts, the interface contains the networks of five backbone to backbone H-bonds between one S9 and another S9 strand. Five additional side-chain to side-chain H-bonds are observed in this interface.

The trimerization interface is formed by the interaction between parts of H1, several loops including L1, L2, L4, and L6 and a β sheet (S2 to S5) from one monomer, and the equivalent regions in an adjacent monomer (Fig. 1c). Upon trimerization, 2938 Å² of surface area becomes buried (Fig. 1d), and among the buried residues, 58% are non-polar groups.

In contrast to the dimerization interface, where no ion pair was observed, a few symmetrically positioned ion pairs (networks) are formed in the trimerization interface; three conserved arginine residues in the L4 loop connecting S4 and H1 (Arg-69, Arg-74, and Arg-77) interact with the acidic residues from the strands S2 and S5 in the next monomer (Fig. 4, a and b). The guanidinium group of Arg-69 makes an ion pair with the carboxyl group of Glu-26. Two residues, Arg-74 and Arg-77, are involved in the five ion-pair network; Glu-63–Arg-74–Asp-116–Arg-77–Asp-118 and Arg-69–Glu-26 are shown in the trimerization interface. a, a close-up view of each ion-pair interaction. Ion-pairs are shown in dotted lines.

b). The importance of these ion pairs in the trimerization of dimers is indicated by the high conservation of the residues that are involved in these interactions in RNase PH family members (Fig. 2).

In addition to the ion-pair formation, several van der Waals interactions and H-bonds contribute to the formation of the outermost monomer.
Perurbng the Oligomeric Interface of RNase PH

**FIG. 5.** Oligomerization states of the mutant *Pa* RNase PHs in solution. *a*, investigation of hexamer formation by gel filtration analysis. The wild-type and mutant *Pa* RNase PH proteins were subjected to a gel filtration column. The oligomeric states in solution are shown for the wild-type (dotted line), R69S (thin line), and R69S/R77S (thick line) mutant proteins. The molecular masses on the top were calculated using a gel filtration standard containing aldolase (158 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The mutant proteins R127A and R77S show the same elution profile as that of the wild-type and were omitted from this diagram for clarity. The elution profiles for the two mutant proteins, R74S and R74S/R77S, are identical to wild type and were omitted from this diagram for clarity. The elution profiles for the two mutant proteins, R74S and R74S/R77S, are identical to wild type and were omitted from this diagram for clarity. The elution profiles for the two mutant proteins, R74S and R74S/R77S, are identical to wild type and were omitted from this diagram for clarity. The elution profiles for the two mutant proteins, R74S and R74S/R77S, are identical to wild type and were omitted from this diagram for clarity.

**Comparison with PNPase Core Domain**—As mentioned above, the *Sa* PNPase core domain that forms a trimeric ring contains two pseudosymmetrically related domains that share about 20% sequence identity with *Pa* RNase PH. Each domain of this central core in PNPase is very similar to the RNase PH monomer (15). The second repeat of PNPase (residues 350–567) shows more similarity to *Pa* RNase PH, with a r.m.s. deviation of 1.4 Å for 200 Cα atoms, whereas the first repeat of the PNPase core is more distantly related, with a r.m.s. deviation of 4.3 Å for 190 Cα atoms. Despite the high similarity in the overall structures between the two proteins, several major differences are observed in the two structures that may contribute to the discrepancies in substrate specificity.

First, each strand that corresponds to the S6 to S7 in *Pa* RNase PH is longer by six residues in *Sa* PNPase. Second, the L3 loop between S3 and S4 is significantly longer in PNPase compared with the equivalent region in *Pa* RNase PH. In addition, this loop in PNPase is bent toward the cleft region containing residues 68–83 of another repeat in the core domain (an equivalent region to the phosphate-binding site in another monomer in RNase PH). Accordingly, the cleft region in the other RNase PH-like repeat adjusts its conformation properly to interact with this loop. Functionally, this conformational difference appears to be significant as this bent loop blocks the entrance of the cleft formed in the first RNase PH-like repeat in *Sa* PNPase. Consequently, as will be described later, this bent loop blocks the entrance, preventing a substrate from threading into this cleft. Consistent with this conformational difference, in PNPase a tungstate ion mimicking the phosphate ion binds to only the second RNase PH-like domain of the central core (15), and in contrast to *Pa* RNase PH, the trimeric ring of *Sa* PNPase has three putative active sites in only one face. Third, the helix H1 in RNase PH is shifted more closely (at least 5 Å) toward the phosphate ion compared with the equivalent region (residues 418–423) in PNPase. This leads the cleft in RNase PH to a more closed form compared with that in PNPase.

Several significant differences are also observed between the phosphate-binding sites of these two proteins. First, only Thr-126 (*Pa* RNase PH), which has been proposed to function as a catalytic residue (15, 17, 18), is a conserved residue that coordinates the phosphate ion in the two proteins. Second, instead of Ser-461 in *Sa* PNPase, the backbone amide of Gly-125 forms an H-bond to the oxygen atom of a phosphate ion. Third, Arg-423 in *Sa* PNPase is equivalent to Arg-87 in *Pa* RNase PH. However, the side chain of Arg-423 is directed away from the tungstate. Instead, an imidazole group of His-427 (Ile-91 in *Pa* RNase PH) is liganded to the tungstate ion (15). And fourth, Ser-423 in *Sa* PNPase is at the position of Arg-127 in *Pa* RNase PH without any interaction with the tungstate ion. Instead of of a wild-type *Pa* RNase PH and are omitted for clarity.

**Fig. 5 continued.** *b*, comparison of melting curves of wild-type and mutant RNase PHs determined by circular dichroism. The spectra for the mutants R69S, R74S, and R77S are identical to that of R69S/R77S, showing the Tm value of 45 °C, whereas a curve of the R127A mutant is superimposed on that of the wild-type protein. All the curves shown here except R127A are measured using a buffer containing 20 mM potassium phosphate, pH 7.4, 150 mM NaCl, and 2 mM DTT. The R127A mutant has been analyzed in the absence and in presence of a phosphate ion. For the analysis of R127A mutant in the absence of a phosphate ion, a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2 mM DTT was used.
Ser-461 interacts with the oxygen atoms in the tungstate ion (15).

Oligomerization States of the Mutant Pa RNase PHs in Solution—Although Pa RNase PH forms a hexameric ring in our structure, other oligomeric forms, including a dimer, are known to be present for Ec RNase PH (11). In addition, another phospholipase exoRNase, PNPase, is in a trimeric form, where the core of each monomer is formed with a dimer of Pa RNase PH, implying that the functionally active form of RNase PH may be a dimer. To understand whether the hexamer is a functionally required form of Pa RNase PH and whether the phosphate-binding site is important for the exoRNase activity, we have mutated several residues at the trimerization interface and at the phosphate-binding site.

To perturb the hexameric ring, we have selected ion pairs in the trimerization interface (Fig. 4, a and b). These ion pairs are highly conserved and symmetrically related, and thus, perturbing an ion pair would provide a 2-fold effect in interrupting the trimerization interface. Three key residues in ion pairs, Arg-69, Arg-74, and Arg-77, were replaced with Ser, and either single or double mutants were created. In the phosphate-binding site, Arg-127 was replaced with Ala, since this conserved arginine residue directly interacts with the phosphate ion.

Initially, to examine whether the mutations have affected the oligomerization state of the Pa RNase PH, the elution profiles of the gel filtration chromatography were compared for the wild-type and mutant Pa RNase PH proteins. The chromatogram of wild-type Pa RNase PH shows a large main peak that corresponds to a hexamer, based on the estimated molecular weight. The single mutation on R69S, R77S, and R127A also showed a single peak at the same position as the wild-type Pa RNase PH (Fig. 5a). However, the R74S mutant exhibited a large peak shift from the peak position of a wild-type Pa RNase PH to the elution volume that is equivalent to the dimeric form (54 kDa) of Pa RNase PH (Fig. 5a). Two double mutants, R69S/R77S and R74S/R77S, have also been eluted to the volume that corresponds to a dimeric Pa RNase PH (Fig 5a). In the case of mutations that dissociate hexameric Pa RNase PH into dimeric form, no other peak at positions that correspond to other oligomeric states has been observed, indicating that disruption of ion pair(s) completely interrupts the trimerization interface. These results suggest that the five-ion-pair network, Glu-63–Arg-74–Asp-116–Arg-77–Asp-118, is critical for the formation of the trimerization interface, and another ion pair, Glu-26–Arg-69, also contributes significantly to the hexameric ring formation.

Conformational Changes upon Interface Mutation—We then investigated whether the dissociation into a dimeric form upon mutation has caused any conformational changes to Pa RNase PH. Circular dichroism (CD) spectroscopy measurement showed that all mutants at the trimerization interface displayed identical patterns of spectra to that of wild-type protein, indicating that no significant changes occurred in the structure of Pa RNase PH upon mutation (Fig. 5b). This suggests that overall, the structures of dimeric mutants are almost identical to that of a wild-type Pa RNase PH. Also CD analysis of the R127A mutant at the phosphate-binding site reveals that the mutant retains the same conformation as that of a wild-type protein (Fig. 5b). This is consistent with the structural data of Ishii et al. (18), where the mutant structure of Arg-126 in Aa...
RNase PH (Arg-127 in Pa RNase PH) is almost identical to that of wild-type Aa RNase PH. We then investigated whether the mutations had affected the thermostability of Pa RNase PH using temperature-dependent CD analysis that was performed in the range from 25 to 90 °C (Fig. 5c). Although the Tm of wild-type Pa RNase PH was 52 °C, the Tm values of all mutants at the trimerization interface had decreased to 45 °C (Fig. 5c). In contrast, a mutant at the phosphate-binding site, R127A, exhibited the same Tm as that of wild-type Pa RNase PH. These data suggest that the intermolecular interactions at the trimerization interface are tightly correlated with the stability of the RNase PH. These data also suggest that although the hexameric ring is not completely dissociated into dimers, the trimerization interface of a ring is partly disrupted upon mutation.

Comparison of Biological Activities—To investigate whether the mutations in the trimerization interface and in the phosphate-binding site affect the biochemical function of Pa RNase PH, phosphorolysis assay of mutants was performed (Fig. 6a). In this assay we have used the precursor tRNA with 10 additional single nucleotides at the 3′ end as a substrate. Fig. 6a shows that, with the exception of the R69S and R127A mutants, all mutants have completely lost their exoRNase activities at 30 and 50 °C. In contrast, the R69S and R127A mutants exhibit full nuclease activity, like wild-type Pa RNase PH, at 30 °C (Fig. 6a). However, when the temperature was increased to 50 °C, the R69S mutant showed slightly decreased activity, which generated incompletely processed products (Fig. 6a).

These results suggest that the dimeric form is insufficient for the exoRNase activity of Pa RNase PH and that the hexameric ring is required for the complete maturation process of the 3′ end of tRNA by Pa RNase PH. The phosphate binding site mutant, R127A, also showed weak and incomplete activity, and only partially cleaved products were formed at 50 °C (Fig. 6a).

To further investigate whether the inactivation by mutations at the trimerization interface is caused by the failure of substrate recognition by RNase PH, we have analyzed the binding ability of wild-type and mutant Pa RNase PHs on precursor tRNA using gel shift assay (Fig. 6b). Initially, the assay was performed using precursor tRNA with 10 extra nucleotides at the 3′ end. Although this substrate binds to Pa RNase PH and could be detected by our system, the intensity of the shifted band was not as strong as we had expected (data not shown). However, we found that the short form of precursor tRNA, with only two additional nucleotides at the 3′ end, binds much more tightly, and we therefore used the shorter tRNA as a substrate. As shown in Fig. 6b, most of the mutant RNase PHs in the trimerization interface, with the exception of the R69S and R127A mutants, cannot bind to precursor tRNA. However, the R69S mutant binds weakly to the substrate at high enzyme concentration (Fig. 6b). These data suggest that the formation of the hexameric ring is an important feature for the binding of substrate. Interestingly, the R77S mutant that can form a hexamer fails to recognize precursor tRNA (Fig. 6b). It is possible that this mutant binds very weakly to precursor tRNA, such that it cannot be detected by our system. Alternatively,
this mutation may not be effective enough to break apart a hexamer into dimers yet is sufficient to disrupt the local structure of a hexameric ring, interfering with the interactions between the substrate and Pa RNase PH. The 7 °C decrease in the T_m values of the R69S and R77S mutants compared with that of the wild-type protein supports the latter hypothesis. The R127A mutant showed slightly decreased binding to precursor tRNA compared with the wild-type protein, suggesting that the side chain of Arg-127 may be involved in the substrate binding (Fig. 6b). However, the intensity of the shifted band did not change significantly, indicating that Arg-127 may not be the critical residue for the recognition of a substrate. The full exoRNase activity for the R127A mutant, observed at 30 °C, supports this idea. However, the significantly decreased nuclease activity of the R127A mutant at 50 °C suggests that the local conformation of this mutant protein might have been changed at the increased temperature. Although the T_m values for the R127A proteins in the presence and in the absence of a phosphate ion are identical to that of the wild-type protein, it is difficult to detect the local structural change from CD analysis.

In the structures of Aa RNase PH proteins, the phosphate-binding site of R126A mutant (R127A in Pa RNase PH) is slightly different from that of the wild-type protein (18); a phosphate ion has shifted by 0.9 Å compared with the wild-type RNase PH with an altered binding mode. As a result, some interactions between the phosphate ion and the liganded residues have been changed. Furthermore, an averaged temperature factor (74.3 Å^2) of a phosphate ion in R126A mutant is relatively higher than that (61.8 Å^2) of a phosphate ion in the wild-type Aa RNase PH. An averaged B factor for whole R126A mutant protein is 52.3 Å^2, and an averaged B factor for whole wild-type Aa RNase PH is 56.3 Å^2. The higher temperature factor for a phosphate ion in R126A mutant suggests that the phosphate ion in the R126A Aa RNase PH is relatively more mobile. Overall, the structural differences in the phosphate-binding site between the wild-type and R126A mutant (R127A in Pa RNase PH) proteins may be correlated with the decreased phosphorolytic activity of R127A Pa RNase PH at the increased temperature.

**DISCUSSION**

In this study we have shown that the Pa RNase PH forms a hexameric ring structure and that ion pairs play a critical role in the trimerization of dimeric Pa RNase PHs. More importantly, we demonstrate that the hexameric ring formation is essential for the binding of precursor tRNA and for exoRNase activity. Previous studies report that the Ec RNase PH can form several different oligomers, depending on protein concentration (11), and thus, it has been unclear whether the formation of a hexameric ring is required for the biological function of RNase PH. Our structural and mutational analyses clearly demonstrate that, at least in the case of Pa RNase PH (which shares 67% identity with Ec RNase PH), the dimer is insufficient for exoRNase activity.

Considering the extensive interactions at the trimerization interface in Pa RNase PH, it is surprising that even a single ion-pair disruption can completely alter the quaternary structure into dimers without affecting the overall monomeric conformation. Although in hyper-thermostable proteins, ion-pair network formations are known to contribute significantly to protein stability (24–26), the stabilizing effect of ion pairs at the mesophilic protein is less clear (27). Our structural studies clearly demonstrate that the formation of ion-pair networks at the oligomeric interface is also important for the stability of a mesophilic protein.

How does RNase PH recognize tRNA precursors or tRNA analogues? It is evident from our studies that two regions, the trimerization interface and the phosphate-binding site at the deep cleft in the dimerization interface, are important for the binding of a substrate and for catalysis. The importance of these regions is further supported by the surface conservation model shown in Fig. 7a, where the two regions are overwhelmingly conserved. Using the structure of yeast tRNA^{5′-P} (PDB code 1EHZ (28)) we have built a complex model between the tRNA substrate and Pa RNase PH such that the single nucleotides region would fit into the deep cleft. Fig. 7b shows a model where the single nucleotides are surrounded by the residues from three helices (H2, H4, and H5), two strands (S8 and S9), and the loop L4 in one monomer and the helix H2 and the loop L3 from another monomer. Because the cleft is narrow the double-stranded part of a tRNA does not fit in. In our model, from the entrance of the cleft (an L4 loop and the H5 helix from one monomer and an L3 loop from another monomer) about five single nucleotides would be sufficient to reach the phosphate ion, which is essential for the exoRNase activity.

If the cleft were deep enough for only five to six single nucleotides, how would a tRNA substrate with a longer single nucleotide strand be digested? Although tRNA with longer single nucleotide strands binds more weakly than tRNA with shorter strands, our data revealed that tRNA with 10 extra single nucleotides could be cleaved by Pa RNase PH. This suggests that the recognition of a single nucleotide region by the residues in the deep cleft in Pa RNase PH must be sequence-independent since the 10 extra single nucleotides in our substrate tRNA do not have a CCA sequence at the 3′ end. Our model suggests that interaction between the entrance of a cleft in the dimer of a Pa RNase PH and the major groove in the double-stranded parts of the tRNA substrate would be required for the binding of tRNA (Fig. 7b). Our model also shows that a few additional regions (the strand S3, two loops L3 and L7, and a turn between H5 and S5) from another dimer that formed on top of the cleft would also participate in the interaction with the double-stranded part of the tRNA, supporting our biochemical data (Fig. 7b). These regions become part of the cleft upon the trimerization of dimeric RNase PHs. Thus, we propose that the additional regions from the adjacent dimer of Pa RNase PH would be required for the proper binding of RNA precursors and for the exoRNase activity.

Although our structural studies provide some clue as to how the hexameric ring might participate in substrate binding, further structural studies of the complex between RNase PH and RNA are essential for the elucidation of the precise interactions and mechanism. Recently, the function of micro RNAs has become more important, and the processing mechanism for these RNAs has drawn significant attention (29). Further systematic structural studies of RNA processing enzymes in both isolated and complexed form with RNAs would help to explain the processing mechanism for several RNAs, including these micro RNAs.

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