Crystal structure of the tRNA processing enzyme

RNase PH from Aquifex aeolicus

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Running title: Crystal structure of RNase PH
Summary

RNase PH is one of the exoribonucleases that catalyze the 3′ end processing of tRNA in bacteria. RNase PH removes nucleotides following the CCA sequence of tRNA precursors by phosphorolysis, and generates mature tRNAs with amino acid acceptor activity. In this study, we determined the crystal structure of *Aquifex aeolicus* RNase PH bound with a phosphate, a cosubstrate, in the active site at 2.3 Å resolution. RNase PH has the typical α/β fold, which forms a hexameric ring structure as a trimer of dimers. This ring structure resembles that of the polynucleotide phosphorylase (PNPase) core domain homotrimer, another phosphorolytic exoribonuclease. Four amino acid residues, Arg86, Gly124, Thr125, and Arg126, of RNase PH are involved in the phosphate binding site. Mutational analyses of these residues showed their importance in the phosphorolysis reaction. A docking model with the tRNA acceptor stem suggests how RNase PH accommodates substrate RNAs.
Introduction

The processing of tRNA precursors into mature tRNAs involves several steps, including the removal of the 5' and 3' extension sequences (1-4). This removal of the 5' and 3' extension sequences is necessary for producing the CCA sequence, which is essential for tRNA aminoacylation and subsequent protein synthesis (2-4). The 5' extension is removed, in a single step reaction, by the ribonucleoprotein RNase P, which is conserved in all organisms (5). On the other hand, the removal of the 3' extension is a more complex process. In archaea and eukaryotes, the 3' extension sequence is removed by RNase Z in a single step reaction (6,7), whereas in bacteria a number of RNases perform this function in multi-step reactions (2-4). In *Escherichia coli*, the proposed 3' processing pathway involves the following steps (2-4). First, an endonucleolytic cleavage occurs downstream of the CCA sequence. RNases E and III have been identified as participating in this first step. Then, the endonucleolytic reaction is followed by the 5'-terminal processing by RNase P, if the 3' trailer sequence of the tRNA precursor is short. However, if the tRNA precursor has a long 3' trailer sequence, an exonucleolytic reaction removes some nucleotides from the 3' end before RNase P processes the 5' end. This exonucleolytic reaction involves RNases PH, T, BN, D, II, and polynucleotide phosphorylase. Finally, these exonucleases remove all of the nucleotides downstream of the CCA sequence; RNase PH and RNase T are usually most effective (8).
In this final trimming, RNase PH catalyzes a 3’ exonucleolytic, phosphorolytic reaction, which needs a phosphate as the cosubstrate, and produces a nucleoside diphosphate (9,10). RNase PH was shown to catalyze the reverse reaction in vitro, the addition of nucleotides to the end of RNA molecules, by using a nucleoside diphosphate as a substrate (10,11). RNase PH reportedly has activity with other small stable RNAs, such as M1, 6S, 4.5S RNA, and tmRNA, in E. coli (12). These stable RNAs, like tRNA, have a common secondary structure of a stem with a 3’ single-stranded tail, which may be related to the substrate specificity of RNase PH (12).

The phosphorolytic ribonucleases can be divided into two major families, the RNase PH family and the polynucleotide phosphorylase (PNPase) family (13,14). The RNase PH homologs are small single-domain proteins and are widely distributed among all three primary kingdoms (13,14). Archaea and eukaryotes have six RNase PH homologs (13-15), and they form a complex as the core of the exosome, which plays the major role in mRNA and rRNA degradation/processing (16).

PNPase, the other type of phosphorolytic ribonuclease, is a large protein consisting of five domains, of which two in the central region are the “PNPase core domains” (13,17). The PNPase core domains are weakly homologous to the RNase PH family members in three regions (the total sequence identity is 20–30%), and are also called “RNase PH domains” (13). In PNPase, the second PNPase core domain is more conserved than the first core.
domain; the catalytic site is conserved only in the second core domain (17). Homologs of PNPase are found in bacteria and eukaryotes, but not in archaea. In bacteria, PNPase nonspecifically removes the 3′ nucleotides of mRNAs (18) and is a component of the degradosome complex (19-21). The crystal structure of Streptomyces antibioticus PNPase has been reported (17). In the PNPase structure, the two PNPase core domains form a homotrimeric ring (17).

In the present study, we determined the crystal structure of RNase PH from Aquifex aeolicus in a complex with a phosphate at 2.3 Å resolution. On the basis of this structure, we carried out site-directed mutagenesis, and determined the crystal structures of the mutants, and thus identified the catalytic site of the RNase PH activity. Based on our docking model with RNA, we discuss how RNase PH stops the 3′ end trimming at the CCA terminus.

Experimental procedures

Cloning, protein expression, and purification of A. aeolicus RNase PH

The A. aeolicus RNase PH gene was amplified by PCR from A. aeolicus genomic DNA, and was cloned into the expression vector pET-26b(+) (Novagen) between the NdeI and SalI sites to construct pET-26b(+) rph. The wild-type RNase PH was overexpressed in E. coli strain BL21-CodonPlus (DE3). Cells were grown in LB medium containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37°C until the A600 was approximately 0.5, and
then isopropyl β-D-thiogalactopyranoside was added to a 1 mM concentration. Cells were
grown at 37°C for 3 more hours and were harvested by centrifugation. The pellet was
resuspended in buffer A [20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl, 1 mM
dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride] and was homogenized by sonication.
The extract was heated for 30 minutes at 70 °C to denature most of the *E. coli* proteins. The
heat-treated fraction was centrifuged and the supernatant fraction was loaded onto a
Q-Sepharose Fast Flow column (Amersham Biosciences) equilibrated with buffer A. The
protein was eluted with a linear gradient of 0.05 – 1 M NaCl. The RNase PH fraction was
pooled and buffer B [buffer A containing 3.5 M (NH₄)₂SO₄] was added so that the final
concentration of (NH₄)₂SO₄ in the protein solution reached 1.5 M; the resultant solution was
then loaded onto a RESOURCE PHE column (Amersham Biosciences). The protein was
eluted with a linear gradient of 1.5 – 0 M (NH₄)₂SO₄, and was purified to more than 95%
homogeneity with a yield of 1-2 mg purified protein from 1 L LB medium, as judged by
SDS-PAGE (Fig. 1).

The R86A mutant gene was made by oligonucleotide-directed mutagenesis, using
the plasmid pET-26b(+)rph as a template, and was ligated into the pET-26b(+) vector. The
expression plasmids containing the T125A or R126A mutant genes were made by the
QuickChange site-directed mutagenesis kit (Stratagene), using pET-26b(+)rph as a template
and following the instructions of the supplier. The nucleotide sequences of the genes were
confirmed by dideoxy sequencing. All of the RNase PH mutants were purified by the same procedure as that used for the wild-type enzyme.

**Crystallization of RNase PH**

Prior to crystallization, the RNase PH fraction was dialyzed against buffer C [20 mM Tris-HCl buffer (pH 8.0) containing 5 mM MgCl₂, 50 mM KCl, and 10 mM NaH₂PO₄], and was concentrated to approximately 1.5 mg/ml by ultrafiltration. Crystallization was accomplished by the hanging drop vapor diffusion method. The crystals were obtained at 20 °C by mixing equal volumes (1:1 each) of the protein solution and a reservoir solution (2 M (NH₄)₂SO₄), and equilibrating the mixture with the reservoir solution. The crystals grew to a maximum size of 0.3 × 0.3 × 0.15 mm³. The crystals belong to the space group P6₃22, with unit cell constants of a = b = 145.4 Å, c = 82.5 Å, and contain one molecule in the asymmetric unit. The selenomethionine (SeMet) derivative was prepared by overexpression of RNase PH in the Met⁻ E. coli strain B834(DE3) with Codon Plus plasmids, and was purified and crystallized by the same procedures used for the native protein. The RNase PH gene for the SeMet derivative was accidentally mutagenized to Q80R, Q120I by a PCR error. However, these mutations did not affect the crystallization and the data collection. All of the mutant proteins were crystallized by the same procedure as that for the wild-type protein.

**Data collection and structure determination**

The multi-wavelength anomalous dispersion (MAD) dataset of the SeMet derivative,
and the native and mutant protein datasets were collected from beamline BL41XU at SPring-8 (Harima, Japan). To reduce the radiation damage during the measurement, the MAD dataset was collected at 30 K using a He cryostream. The data were processed and scaled with the program HKL2000 (22). Six selenium sites were picked out of the seven selenium sites expected in the asymmetric unit, using SnB (23). The selenium parameters were refined and the initial phases were calculated using SHARP (24). The resulting initial phases were extended to 2.9 Å by density modification with the program RESOLVE (25). The atomic model was built using O (26) and was refined using CNS (27). Phasing of the native crystal data set was carried out by molecular replacement using MOLREP (28), with the 2.9 Å SeMet derivative models as a search model. The model of the native crystal was built and refined in a similar manner as that of the SeMet derivative structure. The phasing and refinement statistics are shown in Table 1. The structure of the wild-type protein was used as a starting model for the refinement of all three mutant proteins using CNS (27) (Table 2).

**RNase PH phosphorolysis assay**

For the RNase PH phosphorolysis assay, a tRNA precursor substrate, tRNA^{Val}-CCA-CCTGAGAA (tRNA-CCA-N₈), was prepared by T7 RNA polymerase-directed *in vitro* transcription. The phosphorolysis reactions were performed in 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 5 mM MgCl₂, 10 mM NaH₂PO₄,
300 nM of tRNA precursor substrate, and 30 nM RNase PH. The samples were incubated for 0, 20, 40 or 60 minutes at 65 °C. After extractions with phenol/chloroform, the products were resolved by denaturing 12% polyacrylamide-8 M urea gel electrophoresis and were stained with 0.1% Toluidine blue.

**Assay for tRNA binding**

A tRNA precursor substrate, tRNA_{Val}^{Val}-CCA-CC (tRNA-CCA-N_2), was prepared by the same procedure as that for tRNA-CCA-N_8. The tRNA binding reactions were performed at 37 °C in 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 5 mM MgCl_2, ±10 mM NaH_2PO_4, 35 nM of ^32^P-labeled tRNA precursor substrate, and 44, 88, or 176 nM of the proteins. After an incubation for 10 minutes, the samples were resolved by 5% polyacrylamide gel electrophoresis, and were visualized by autoradiography of the gel.

**Results and Discussion**

**Overall Structure of RNase PH**

The crystal of *A. aeolicus* RNase PH (28 kDa) belongs to the space group *P*6_3*2*, and contains a single subunit in the asymmetric unit. The crystal structure of RNase PH was solved by the MAD method at 2.3 Å resolution. The final model of RNase PH consists of all of the residues, except for the N terminal methionine residue, and has an R_{work} of 23.3% and an R_{free} of 27.5% (Table 1). Six subunits, which are related by crystallographic symmetry,
form a hexameric ring structure as a trimer of dimers (Figs. 2A, 2B). This ring has 32
symmetry, and the molecular 3-fold axis corresponds to the crystallographic 3-fold axis (Fig.
2A). Actually, *A. aeolicus* RNase PH also forms a hexamer in solution, as the average
molecular weight of the RNase PH sample was estimated to be about 170 kDa (6.1 × 28 kDa)
by the ultracentrifugation analysis (data not shown). On the other hand, the *E. coli* RNase PH
tends to self-associate and to form multimers of various sizes (10).

The overall architecture of RNase PH is a βαβα fold (Fig. 2C). The dimerization
interface consists of three parts of the molecules: the N-terminus of helix α6 forms the outer
face of the molecule, the N-terminus of helix α3 is near the center of the ring, and the
β-strand β9 is between these α-helices (Figs. 2B, 2C). These α3, α6, and β9 structures
interact with those of the adjacent subunit, respectively, and therefore, the antiparallel β-sheet
consisting of β6-β9 extends to an adjacent subunit (Figs. 2B, 2C). The trimerization interface
is composed of the loop connecting β1 and β2, the region through η1 to α2, and half of the
6-stranded β-sheets (β3-β5) (Figs. 2A, 2C). On the other hand, the other half of the β-sheet
(β1-β2 and β12) is exposed to the solvent and forms an intermolecular cleft (Figs. 2A, 2C).
The outer face of the hexameric ring consists of one long α-helix (α6) running across the
intermolecular β-sheet, as mentioned above (Figs. 2A, 2B). The buried surface area of the
dimer is 1460 Å², and that of the trimer is 1440 Å². The central hole of the hexameric ring is
made up of the α2 helix (Figs. 2A, 2C), but this region is partially disordered. The diameter
of the central hole is about 5 Å, which is smaller than that of a nucleotide.

**Structural comparison between RNase PH and PNPase**

The crystal structure of *S. antibioticus* PNPase, which has the duplicated “PNPase core domains” and the three accessory domains, was previously determined (17). In the PNPase structure, the two PNPase core domains, which are related by pseudosymmetry, homotrimerically form the core ring, with the other domains placed on its upper and lower faces (17). The overall structure of *A. aeolicus* RNase PH is similar to that of the core-domain ring of *S. antibioticus* PNPase. The homodimeric structure of *A. aeolicus* RNase PH corresponds to the pseudosymmetric structure of the duplicated PNPase core domains. The *A. aeolicus* RNase PH structure superimposes well on the second PNPase core domain structure, with a root-mean-square deviation (r.m.s.d.) of 1.5 Å over 198 corresponding Cα atoms (Fig. 3).

There are several differences between the two structures. First, helix α4 of *A. aeolicus* RNase PH is much longer than the corresponding helix α10 of PNPase (Fig. 4). Second, the loop connecting β6 and β7 of *A. aeolicus* RNase PH is shorter than that of the PNPase. Third, *A. aeolicus* RNase PH has a 20-residue extension at the C-terminus (β10, β11, and β12) (Figs. 3, 4). The three C-terminal β-strands of *A. aeolicus* RNase PH form the cleft between the dimers in the trimeric arrangement (Figs. 2A, 4).

Furthermore, a Dali search (29) of the Protein Data Bank with the coordinates of *A.
aeolicus} RNase PH revealed that its N-terminal domain (residues 24-140 and 200-240) shares structurally-homologous parts with homoserine kinase (30) and ribosomal protein S5 (31). According to the SCOP classification (32), homoserine kinase and ribosomal protein S5 have the same 2-layer ββαβα sandwich fold, named the ribosomal protein S5 domain 2-like fold. The ribosomal protein S5 domain 2-like fold superfamily includes the PNPase core domain, the RNase P protein, and ribosomal protein S9. The structural similarity between the PNPase core domain and the RNase P protein was previously mentioned (17).

**Phosphate binding**

In the present electron density map for the RNase PH crystal, a strong electron density peak (>5σ) was observed and modeled well as a bound phosphate. It is possible that the bound phosphate corresponds to the inorganic phosphate or the phosphodiester phosphate of RNA in the phosphorolysis reaction. This phosphate binding site is at the bottom of a cleft formed by the helices α3–α5 and the β-sheets β6–β9 (Figs. 2C, 5A). As the RNase PH subunit has one phosphate binding site, the hexameric ring has three active sites on both sides (Fig. 2A). The phosphate is surrounded by the N-terminal parts of helices α3 and α4 (Fig. 5A). The phosphate is accommodated in a pocket formed by the side chains of Arg86 (α3), Thr125 (α4), and Arg126 (α4) and the main chain NH group of Gly124; one oxygen atom interacts with the Arg86 Nζ, the Thr125 Oγ, and one water, two interact with the Arg126 Ne and Nζ, and one interacts with the Gly124 NH (Fig. 5A). The four phosphate-interacting
amino acid residues are strictly conserved in the bacterial RNase PH sequences (Fig. 4).

As shown in Fig. 5B, the structure of the phosphate binding site on RNase PH superimposes well on that of the corresponding site on the second PNPase core domain, which is partially occupied by a phosphate analog, tungstate (17). Four residues, His427, Ser461, Thr462, and Ser463, of \textit{S. antibioticus} PNPase interact with the tungstate (Fig. 5B) (17). In PNPases, His427, Ser461, and Ser463 are strictly conserved. On the other hand, the position corresponding to Thr462 is either Ser or Thr, but Ser is more common than Thr (Fig. 4). In contrast, the phosphate binding site consists of four residues, Arg86, Gly124, Thr125, and Arg126, in \textit{A. aeolicus} RNase PH (Fig. 5A). Therefore, the corresponding active sites of \textit{S. antibioticus} PNPase and \textit{A. aeolicus} RNase PH share only one Thr residue (Thr462 and Thr125, respectively). In addition to the four tungstate binding residues listed above, Arg423 of \textit{S. antibioticus} PNPase is conserved in other PNPases. Interestingly, the Ca position of Arg423 in \textit{S. antibioticus} PNPase is similar to that of Arg86 in \textit{A. aeolicus} RNase PH, whereas the Arg423 side chain is directed away from the tungstate (Fig. 5B). Therefore, it may be possible that Arg423 is also involved in the phosphorolytic reaction by \textit{S. antibioticus} PNPase. Actually, in the previous study of \textit{E. coli} PNPase, the phosphorolytic activity was completely abolished by the double Asp mutation of Arg398-Arg399 (33), corresponding to Arg422-Arg423 of \textit{S. antibioticus} PNPase and to Gly85-Arg86 of \textit{A. aeolicus} RNase PH, respectively.
RNase PH phosphorolysis assay

To investigate whether the phosphate interacting residues of RNase PH are critical for the phosphorolytic exoribonuclease activity, we constructed three alanine-substituted mutants, R86A, T125A, and R126A. In this study, we used tRNA-CCA-N₈ as the tRNA precursor analog, and detected the processing products directly by denaturing polyacrylamide gel electrophoresis. *A. aeolicus* RNase PH rapidly digested the 3’ extended tRNA precursors, and generated the mature-size tRNA within 20 minutes (Fig. 6A). The three mutants exhibited the activity to different extents, with rather high enzyme concentrations (Fig. 6A). The R86A mutant showed no detectable products even after a 60-minute incubation, and thus this mutant is completely inactive. The T125A mutant has marginal activity, producing RNA digests a few nucleotides shorter than tRNA-CCA-N₈ after a 60-minute incubation. The R126A mutant is significantly more active than the other two mutants, but cannot cleave the precursor to the mature size even after a 60-minute incubation. Therefore, the activities of the T125A and R126A mutants are much lower than that of the wild type. These results show that the three strictly conserved residues, Arg86, Thr125, and Arg126, are very important for the phosphorolytic activity of the bacterial RNase PH.

Gel shift and crystallographic analyses of RNase PH mutants

We then performed two other experiments to investigate how these mutations affect the activity. To explore their effects on the tRNA binding ability, we performed gel shift
assays using the tRNA precursor (tRNA-CCA-N₂) (Fig. 6B). The wild-type RNase PH caused the gel shift of the tRNA precursor in the presence of the phosphate ion (lanes 6-8, Fig. 6B). The amount of the gel-shifted tRNA precursor is smaller than that in the absence of the phosphate ion (lanes 2-4), probably because of electrostatic repulsion between the enzyme-bound phosphate ion and the phosphate group of the tRNA precursor. The three mutations had different effects on the tRNA precursor gel shift. First, the R86A mutant exhibited nearly the same level of gel-shifted tRNA as that of the wild type (lanes 9-11). Therefore, the replacement of Arg86 by Ala does not affect the tRNA binding ability. By contrast, the T125A mutant gel-shifted more of the tRNA precursor than the wild type (lanes 12-14). The observed increase in the RNA binding ability by the T125A mutation might be due to a decrease in the putative electrostatic repulsion between the phosphate ion and the RNA phosphate group. On the other hand, the R126A mutant bound less RNA than the wild type (lanes 15-17). The low tRNA binding ability of the R126A mutant may suggest that Arg126 is involved in RNA binding, rather than phosphate ion binding.

Furthermore, we have determined the crystal structures of the three mutants at 2.3 Å resolution. These mutants were crystallized under the same conditions used for the wild type, and the overall structures were the same as that of the wild type, with r.m.s.d. values of 0.18, 0.26, and 0.29 for R86A, T125A, and R126A respectively. In the present electron density maps for each of the three mutant crystals, the same electron density peaks were present at
the phosphate binding site as that in the wild-type crystal. However, the phosphate binding modes slightly differ from each other (Figs. 7A, 7B, 7C). In the R86A mutant, the phosphate position is the same as that of the wild type (Fig. 7A). In addition, the RNA binding ability of the R86A mutant is similar to that of the wild type (Fig. 6B). Hence, the replacement of Arg86 by Ala has hardly any effect on the phosphate and RNA binding. Nevertheless, the R86A mutant showed no phosphorolytic activity (Fig. 6A). Therefore, Arg86 may play an important role in the phosphorolysis reaction, although it is possible that a rearrangement of the phosphate binding site might occur when the enzyme binds RNA.

In the T125A mutant, the phosphate position is 0.39 Å away from that of the wild type, and the interaction between Arg86 and the phosphate ion is abolished (Fig. 7B). On the other hand, the side chain of Arg126 has moved together with the phosphate ion. These effects of the T125A mutation on the active site structure may be related to the decrease in the phosphorolytic activity and the increase in the tRNA precursor binding. In the R126A mutant, the phosphate position is shifted by 0.89 Å as compared with the wild type (Fig. 7C). As a result, the phosphate ion has no interaction with Arg86, but remains within range of the interaction with Thr125. Furthermore, the phosphate ion interacts with the side chain of Asn188, which is conserved in bacterial RNases PH. The phosphorolysis activity of the R126A mutant is lower than that of the wild type, but is much higher than those of the other two mutants (Fig. 6A). On the other hand, the R126A mutant has slightly lower binding
affinity for the tRNA precursor than that of the wild type (Fig. 6B).

The structural and mutational analyses of the present study suggest the following mechanisms for the phosphorolytic reaction by RNase PH. First, the Thr125 and Arg126 residues bind the phosphate ion (Figs. 5A, 7). The Arg126 residue may also bind the diester phosphate group of the tRNA precursor. The RNA binding might change the phosphate ion binding residues from Thr125/Arg126 to Thr125/Asn188, as in the R126A mutant (Fig. 7C). Then, the Arg86 is likely to interact with the two substrates for the phosphorolytic reaction.

RNA interaction model

In the crystal structure, the phosphate binding site is located in the bottom of a deep cleft (Figs. 2A, 2B). The entrance of the cleft is narrowed by helix α1 and the following loop (residues 48-56) of the adjacent subunit related by the two-fold symmetry. This overhanging region at the entrance allows only the 3′ terminal single-stranded region of the tRNA precursor to enter the active site (Figs. 2B, 2C). Although the overhanging region has relatively high B-factors (about 65 Å²), we postulated that the narrow entrance of the active site cleft can discriminate single-stranded RNA from double-stranded RNA, and constructed a local docking model with a tRNA acceptor stem derived from the yeast tRNA^{Phe} (PDB ID 1EHZ (36)). When the double-stranded acceptor stem of the tRNA is located closest to the cleft entrance, the four single-stranded nucleotides are long enough to reach the active site (Figs. 8A, 8B). If the 3′ extension is shorter than four nucleotides, then it seems difficult for
the 3’ end nucleotide to reach the active site. Therefore, the present model may be relevant to
the mechanism, in that RNase PH can remove non-base paired nucleotides from a tRNA
precursor with a long 3’ extension until it reaches the CCA terminus. RNase PH may process
other RNAs with the same structural features (a stem followed by a single-stranded sequence
at the 3’ end) in a similar manner.

In our model, the dimer of RNase PH only interacts with the tRNA acceptor stem,
and the other parts of the tRNA remain unbound. RNase PH has no other RNA binding
domain, while PNPase has the KH and S1 domains. Therefore, the only possibility for any
further interaction of RNase PH with the RNA substrate is that other RNase PH dimers in the
hexameric ring interact with some distant part (e.g. the anticodon arm) of the tRNA precursor.
It might also be possible that the tRNA precursor interacts with some other RNA binding
protein that associates with RNase PH (perhaps with other RNase PH subunits in the
hexameric ring). On the other hand, the hexamer of RNase PH might dissociate into dimers,
as in the case of *E. coli* RNase PH (10), upon binding with the tRNA precursor. To reveal the
precise mechanism of the tRNA 3’ end processing by bacterial RNase PH, further studies,
such as the structure determination of a complex of RNase PH and an RNA substrate, are
necessary.
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References


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Footnotes

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID code 1UDN, 1UDO, 1UDP, 1UDS).

The abbreviations used are: PNPase, polynucleotide phosphorylase; SeMet, selenomethionine.
Figure legends

**Figure 1  Purification of RNase PH.**

Purification of RNase PH was monitored by SDS-PAGE, and gels were stained with Coomassie Brilliant Blue. *Lane 1*, protein size markers of the masses indicated; *Lane 2*, RESOURCE PHE combined fraction.

**Figure 2  Ribbon diagram displaying the overall structure of *A. aeolicus* RNase PH.**

*(stereo views).* (A) Top view of the RNase PH hexamer. The 3-fold axis is perpendicular to the paper. The six subunits are colored differently, and the phosphates are colored red. (B) Side view of the RNase PH hexamer. The 3-fold axis is parallel to the paper. The coloring scheme is the same as in (A). (C) The RNase PH subunit structure, in the same orientation of the monomer colored cyan in (B). The α and 3₁₀ helices are colored yellow and orange, respectively. The β strands are colored blue. The phosphate is colored red. All of the graphic figures in the present paper were drawn by using DINO (http://www.dino3d.org).

**Figure 3  Comparison of RNase PH and PNPase structures.**

The *A. aeolicus* RNase PH (green) structure superimposes well on the *S. antibioticus* second PNPase core domain structure (blue), with a root-mean-square deviation of 1.5 Å over 198 corresponding Cα atoms (stereo view). The structure comparison was made using Secondary Structure Matching (SSM) (http://www.ebi.ac.uk/msd-srv/ssm/).
Figure 4  Structure-based sequence alignment between RNases PH and the second core domain of PNPases.

Only the *A. aeolicus* RNase PH and *S. antibioticus* PNPase sequences are shown. Highly conserved residues and semi-conserved residues within each enzyme are colored red and green, respectively; blue rectangles show identical residues between the two enzymes.

Secondary structures are colored corresponding to those in Figure 1C.

Figure 5  Stereo view of the phosphate binding site.

(A) The phosphate binding site of *A. aeolicus* RNase PH. The side chains of Arg86, Glu124, Thr125, and Arg126, which interact with the phosphate, are depicted as a ball and stick representation. The carbon, nitrogen, oxygen, and phosphate atoms are colored gray, blue, red, and green, respectively. (B) Superposition of the phosphate binding residues of *A. aeolicus* RNase PH (green) and the tungstate binding residues of *S. antibioticus* PNPase (blue). The phosphate and tungstate are colored red and magenta, respectively.

Figure 6  Mutational analysis of the RNase PH.

(A) Denaturing PAGE analysis of the RNase PH phosphorolytic activity. Lane 1, tRNA-N8 as a marker; lanes 2-5, wild-type RNase PH; lanes 6-9, R86A mutant; lanes 10-13, T125A mutant; lanes 14-17, R126A mutant; lane 18, tRNA as a marker. The reactions were carried out for different incubation times as indicated (0, 20, 40, 60 minutes, respectively). The wild-type RNase PH generated the mature-size tRNA within 20 minutes. On the other hand,
the R86A mutant showed no detectable activity, and the T125A and R126A mutants showed faint or weak activities. (B) The gel shift assay for tRNA binding. The tRNA binding affinities of the wild type and three mutants of RNase PH were analyzed by incubating 125, 250, and 500 ng of the proteins with 100 ng of \(^{32}\)P-labeled tRNA. All of the mutants were analyzed in buffer containing phosphate. Lanes 1 and 5, only tRNA in the absence or presence of phosphate, respectively; lanes 2-4, wild-type in the absence of phosphate; lanes 6-8, wild-type in the presence of phosphate; lanes 9-11, R86A mutant; lanes 12-14, T125A mutant; lanes 15-17, R126A mutant. RNase PH binds tRNA-N2 more tightly in the absence of phosphate than in the presence of phosphate. The three mutants had different tRNA binding abilities. The binding ability of the R86A mutant is nearly the same as that of the wild type. On the other hand, the binding ability of the T125A mutant is stronger than that of the wild type, and the binding ability of the R126A mutant is slightly lower than that of the wild type.

**Figure 7  Comparison of the wild-type and three mutant structures.**

Superposition of the phosphate binding residues of the wild-type (gray) and the mutant proteins (red, A: R86A mutant, B: T125A mutant, and C: R126A mutant). The overall structures of these variants are the same as that of the wild type. However, the phosphate binding modes slightly differ among them. In the R86A mutant, the phosphate binding position is the same as that of the wild type. On the other hand, in the T125A and R126A
mutants, the phosphate is 0.39 Å and 0.89 Å away from the position in the wild type, respectively.

**Figure 8  Docking model of RNase PH with RNA.**

(A) Top view of the surface model. The subunits of RNase PH are colored according to those in Figure 1A. RNA and phosphate are colored yellow and red, respectively. Only one RNA molecule is shown. The docking model was constructed manually, avoiding steric hindrance.

(B) Magnified view of the area indicated with a black rectangle in (A). The coloring scheme is the same as in (A).
### Table I. Summary of data collection, phasing, and refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>SeMet</th>
<th>Native</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak</td>
<td>Edge</td>
</tr>
<tr>
<td><strong>Data Collection</strong></td>
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<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.9791</td>
<td>0.9793</td>
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<tr>
<td>Resolution (Å)</td>
<td>50-3.5</td>
<td>50-3.5</td>
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<tr>
<td><strong>Cell constants</strong></td>
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<tr>
<td>$a = b$ (Å)</td>
<td>142.23</td>
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</tr>
<tr>
<td>c (Å)</td>
<td>86.53</td>
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</tr>
<tr>
<td>Unique reflections</td>
<td>6,505</td>
<td>6,620</td>
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<tr>
<td>Redundancy</td>
<td>9.1</td>
<td>9</td>
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<tr>
<td>Completeness (%)</td>
<td>94.7 (95.2)</td>
<td>94.7 (92.2)</td>
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<tr>
<td>$I / \sigma(I)$</td>
<td>33.3 (15.2)</td>
<td>30.3 (9.5)</td>
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<tr>
<td>$R_{sym}$ (%)</td>
<td>7.6 (13.4)</td>
<td>7.7 (17.9)</td>
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<tr>
<td><strong>Phasing</strong></td>
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<tr>
<td>Phasing power</td>
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<tr>
<td>iso (centric)</td>
<td>2.83 (1.70)</td>
<td>1.18 (0.68)</td>
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<tr>
<td>ano</td>
<td>2.83</td>
<td>3.14</td>
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<tr>
<td>FOM acentric/centric</td>
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<td></td>
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<td><strong>Refinement</strong></td>
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<td>$R_{work}$ (%)</td>
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</tr>
<tr>
<td>$R_{free}$ (%)</td>
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</tr>
<tr>
<td>r.m.s.d. bond length (Å)</td>
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<tr>
<td>r.m.s.d. bond angles (')</td>
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</tbody>
</table>

*R_{sym}=\sum |I_{avg}-|I_i| / \sum |I_i|, \text{ } +R_{cullis}=\sum \|F_{PH}+F_{PH}|-F_{H}(\text{calc})| / \sum |F_{PH}|

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Table II. Summary of data collection and refinement statistics

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<th>R86A</th>
<th>T125A</th>
<th>R126A</th>
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<tbody>
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<td>Wavelength (Å)</td>
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<td>Resolution (Å)</td>
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<td><strong>Cell constants</strong></td>
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<td><em>a = b</em> (Å)</td>
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<td>144.81</td>
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<td>c (Å)</td>
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<td><strong>Unique reflections</strong></td>
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<td>13.2</td>
<td>13.2</td>
<td>13.2</td>
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<tr>
<td><strong>Completeness (%)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>All data (last shell)</td>
<td>99.5 (99.3)</td>
<td>99.4 (98.8)</td>
<td>98.6 (98.3)</td>
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<tr>
<td><em>I/σ(I)</em> All data (last shell)</td>
<td>19.9 (2.0)</td>
<td>23.0 (2.0)</td>
<td>26.2 (2.7)</td>
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<tr>
<td><strong>Rsym</strong> (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All data (last shell)</td>
<td>7.4 (46.7)</td>
<td>7.3 (48.1)</td>
<td>5.7 (31.7)</td>
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<tr>
<td><strong>Refinement</strong></td>
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<tr>
<td>Rwork (%)</td>
<td>22.5</td>
<td>23.1</td>
<td>22.1</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>26.4</td>
<td>26.3</td>
<td>25.5</td>
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<tr>
<td>r.m.s.d. bond length (Å)</td>
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<td>0.01</td>
<td>0.01</td>
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<td>r.m.s.d. bond angles ()</td>
<td>1.70</td>
<td>1.60</td>
<td>1.60</td>
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</table>

*Rsym = \frac{\sum |I_{avg} - I_i|}{\sum I_i}, \ \ \ \ \ \ \ \ \ \ ^{\dagger}R_{cullis} = \frac{\sum \|F_{PH}^+ + F_{PH}^- - F_{H}(calc)\|}{\sum \|F_{PH}\|}
Figure 1
Figure 2
Figure 3
<table>
<thead>
<tr>
<th>Aa_RNasePH</th>
<th>Sa_PNPase</th>
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<tbody>
<tr>
<td>MRSDGPKEDQLPVSIQRFDEYPFESCISIERTKVICTASVENVFPWML...KGKGQ</td>
<td>RMIDGVSVDITLAAEVEAPRVSFAQSLFEMEQILGVTTNLMLREMQQGLDSTPVT</td>
</tr>
</tbody>
</table>

**Figure 4**
Figure 5
Figure 6
Figure 7
Figure 8