Functional implication of archaeal homologues of human RNase P protein pair Pop5 and Rpp30

Received May 10, 2015; accepted June 9, 2015; published online July 7, 2015

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PhoPop5 and PhoRpp30 in the hyperthermophilic archaeon *Pyrococcus horikoshii*, homologues of human ribonuclease P (RNase P) proteins hPop5 and Rpp30, respectively, fold into a heterotetramer [PhoRpp30–(PhoPop5)₂–PhoRpp30], which plays a crucial role in the activation of RNase P RNA (PhoRNA). Here, we examined the functional implication of PhoPop5 and PhoRpp30 in the tetramer. Surface plasmon resonance (SPR) analysis revealed that the tetramer strongly interacts with an oligonucleotide including the nucleotide sequence of a stem-loop SL3 in PhoRNA. In contrast, PhoPop5 had markedly reduced affinity to SL3, whereas PhoRpp30 had little affinity to SL3. SPR studies of PhoPop5 mutants further revealed that the C-terminal helix (α 4) in PhoPop5 functions as a molecular recognition element for SL3. Moreover, gel filtration indicated that PhoRpp30 exists as a monomer, whereas PhoPop5 is an oligomer in solution, suggesting that PhoRpp30 assists PhoPop5 in attaining a functionally active conformation by shielding hydrophobic surfaces of PhoPop5. These results, together with available data, allow us to generate a structural and mechanistic model for the PhoRNA activation by PhoPop5 and PhoRpp30, in which the two C-terminal helices (α 4) of PhoPop5 in the tetramer whose formation is assisted by PhoRpp30 act as binding elements and bridge SL3 and SL16 in PhoRNA.

Keywords: archaea/protein–RNA interaction/
Pyrococcus horikoshii/ribonuclease P/surface plasmon resonance.

Abbreviations: 3-D, three-dimensional; PhoRNA, ribonuclease P RNA from *P. horikoshii*; pre-tRNA, precursor tRNA; RNase P, ribonuclease P; RNP, ribonucleoprotein; RRM, RNA recognition motif; SL3, stem-loop containing P3 helix; SL16, stem-loop containing P16 helix; SPR, surface plasmon resonance.

Ribonuclease P (RNase P) is a ubiquitous *trans*-acting ribozyme that catalyses the processing of 5' leader sequences from tRNA precursors (pre-tRNA) and other noncoding RNAs in all living cells (1, 2). In contrast to eubacterial RNase P RNAs, the RNA components in archaea and eukaryotes alone have little catalytic activity *in vitro* and function in cooperation with protein subunits in substrate recognition and catalysis (3). Hence, archaeal and eukaryotic RNase Ps may serve as a model ribonucleoprotein (RNP) for studying how a functional RNA can be activated by protein cofactors and how the RNP enzymes catalyse biological processes.

We earlier found that RNase P RNA (PhoRNA) and five proteins in the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 reconstituted RNase P activity that exhibits enzymatic properties like those of the authentic enzyme (4, 5). The *P. horikoshii* RNase P proteins were designated PhoPop5, PhoRpp21, PhoRpp29, PhoRpp30 and PhoRpp38, according to their sequence homology with the human RNase P proteins hPop5, Rpp21, Rpp29, Rpp30 and Rpp38, respectively (6). Biochemical and structural studies revealed that PhoPop5 and PhoRpp21 form a complex with PhoRpp30 and PhoRpp29, and the resulting complexes, PhoPop5–PhoRpp30 and PhoRpp21–PhoRpp29, are involved in activation of the C- and S-domains, respectively (7–9). Functional reconstitutions of the RNA component and individual proteins, and the cooperative of archaeal homologues of human RNase P protein pairs of Pop5 with Rpp30 and Rpp21 with Rpp29 were reported on other archaeal RNase Ps (10–13).

The X-ray structure shows that PhoPop5 and PhoRpp30 fold into a heterotetramer [PhoRpp30–(PhoPop5)₂–PhoRpp30], in which a homodimer of PhoPop5 sits between two PhoRpp30 monomers (Fig. 1). PhoPop5 dimerizes through hydrogen bonding interaction from the loop between α 1 and α 2 helices. The reconstituted particle containing the PhoPop5 mutant termed Δ L43–48, in which the α 1– α 2 loop in PhoPop5 was deleted, had significantly reduced pre-tRNA cleavage activity (7, 14). Furthermore, reconstitution experiments indicated that deletion of the C-terminal helices α 4 and α 5 (3₁₀-helix) significantly influenced the pre-tRNA cleavage activity, whereas that of α 5 had little effect on this activity (14). These results indicate that the heterotetrameric structure is essential for activation of PhoRNA, and that C-terminal helix α 4 in PhoPop5 plays a crucial role in the activation of PhoRNA. Recently, we found that PhoRNA mutants Δ P3 and Δ P16, in which stem-loops SL3 and SL16 containing P3 and P16 in PhoRNA were deleted, respectively,

Fig. 1 The crystal structure of the *PhoPop5–PhoRpp30* complex. The crystal structure of the *PhoPop5–PhoRpp30* complex (Protein Data Bank accession code 2CZV) was drawn using Pymol (<http://www.pymol.org>). N and C indicate the N- and C-termini of *PhoPop5*, respectively. The C-terminal helices $\alpha 4$ and $\alpha 5$ are highlighted in red, and the $\alpha 1$ – $\alpha 2$ loop in *PhoPop5* is green. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

had little ability to bind *PhoPop5* and *PhoRpp30*, suggesting that the *PhoPop5–PhoRpp30* complex specifically recognizes SL3 and SL16 (15). Abundant evidence demonstrates that the tetrameric formation of the archaeal RNase P protein pair Pop5 and Rpp30 is indispensable for the catalytic activity of archaeal RNase Ps, but the mechanism behind this remains unclear.

To address this issue, surface plasmon resonance (SPR) analysis using Biacore X-100 was employed to analyse interactions of *PhoPop5*, its mutants, and *PhoRpp30* with SL3 in *PhopRNA*. Furthermore, the functional implication of *PhoRpp30* in the tetramer was investigated by gel filtration and kinetic analysis of the reconstituted particles containing its mutants. The results indicated that the C-terminal helix ($\alpha 4$) in *PhoPop5* functions as a molecular recognition element for SL3, whereas *PhoRpp30* primarily serves as a molecular chaperone for *PhoPop5* to form a functionally active structure in the tetramer. On the basis of these results, we present a three-dimensional (3-D) model, in which the two C-terminal helices ($\alpha 4$) of *PhoPop5* in the tetramer bridge SL3 and SL16, which suggests a molecular basis for the *PhopRNA* activation by *PhoPop5* and *PhoRpp30*.

Materials and Methods

Materials

Ex Taq DNA polymerase and the DNA ligation kit were purchased from Takara Bio (Shiga, Japan). SA sensor chips and Biacore consumables were obtained from GE Healthcare. The 5'-biotinylated oligonucleotides and template oligonucleotides for RNA synthesis were purchased from Eurofins MWG Operon (Ebersberg, Germany). All other chemicals were of analytical grade for biochemical use.

Preparation of proteins and RNAs

Five RNase P proteins (*PhoPop5*, *PhoRpp21*, *PhoRpp29*, *PhoRpp30* and *PhoRpp38*), *PhopRNA* and pre-tRNA^{Tyr} in *P. horikoshii* were

prepared as described previously (4, 5). The deletion mutant $\Delta L43$ –48, in which the residues 43–48 including Glu44 and Glu48 in *PhoPop5* were deleted, and the C-terminal truncated mutant ($\Delta 14C$) of *PhoPop5* were prepared as described previously (7, 14). Mutants of *PhoRpp30*, in which Arg90, Arg107, Lys123, Arg176, Asp180 and Lys196 in *PhoRpp30* were individually replaced by Ala, were prepared as described previously (16).

Biosensor experiments

All SPR experiments were performed with a Biacore X-100® instrument (GE-Healthcare Biacore, Uppsala, Sweden). The running buffer composition was 20 mM phosphate buffer (pH 7.2), 500 mM NH₄Cl and 0.05% Tween20. 5'-labeled biotin-oligonucleotides (Fig. 2A) were immobilized on SA sensor chips. These SA sensor chips had been preconditioned by triplicate injections of 50 mM NaOH and 2 M NaCl, each with a contact time of 60 s. Before immobilization, the single-strand oligonucleotides, diluted at a low concentration of running buffer, were heated at 90°C for 1 min and put on ice before injection onto the SA sensor chip at 10 μ l/min at 25°C to generate 100–150 RU on the surface. RNA chain Ta-SL3 with stem-loop SL3 was prepared by *in vitro* transcription and preformed at 500 nM in running buffer, with heating at 95°C for 15 min and then slowly allowing cooling to 4°C. The product was used for injection onto the SA sensor chip to reach a density of 200 RU. Proteins were serially diluted in running buffer to the final concentrations indicated in Figs 2B–D and injected at a flow rate of 25 μ l/min for 35–60 s. The responses were also monitored during a 3- to 6-min dissociation phase. After this dissociation step, samples were kept in 50 mM NaOH in water, each for 60 s, to remove any residual protein and Ta-SL3. Each concentration series was run in duplicate (and sometimes in triplicate or quadruplicate). All binding data were collected at 25°C.

Data processing

Data processing and analysis were performed using Biacore X-100® software package in BiaEval 4.1. The responses were double-referenced by subtracting from the signal on the 5'-labeled biotin-oligonucleotide surface, both the signal recorded on the reference surface and the signal of a blank injection with the running buffer (17). Binding experiments were performed to determine the association and dissociation rate constants, k_{on} and k_{off} , respectively, for the complex formation. The dissociation equilibrium constant, K_D , was calculated as k_{off}/k_{on} . The sensorgrams were fitted assuming simple reversible bimolecular reactions as described by Palau and Primo (18). When sensorgrams could not be fitted, the steady-state values were plotted against the peptide concentration and the K_D values were determined by non-linear regression analysis of the steady-state values using a single site model: $[Comp] = R_{max-exp} \times [Pro]/(K_D + [Pro])$, where $R_{max-exp}$ is the maximal binding capacity, $[Comp]$ the concentration of complex at a steady state, and $[Pro]$ the concentration of the protein.

Assay for pre-tRNA cleavage activity

The RNase P activity for the reconstituted particles was analysed in the reconstitution buffer, 50 mM Tris–HCl (pH 7.6) containing 50 mM MgCl₂, 600 mM NH₄OAc and 60 mM NH₄Cl, principally as described previously (15). To examine the kinetic constants of the RNase P activity of the reconstituted particles, the pre-tRNA cleavage activity was analysed in the presence of *PhoRpp30* or its mutants (5 pmol), the other four proteins (each 5 pmol), *PhopRNA* (5 pmol) and pre-tRNA^{Tyr} (1.88–19.4 μ g) for 15 min. As for the analysis of the stoichiometry, various amounts of *PhoPop5* and *PhoRpp30* or *PhoRpp21* and *PhoRpp29* were incubated with the reconstituted particles containing *PhopRNA* (10 pmol) and the other three proteins (each 10 pmol) for 15 min at 75°C. The reactions were stopped by adding phenol, and the reaction products were separated on 10% polyacrylamide denaturing gels in TBE buffer (900 mM Tris-borate containing 10 mM EDTA) at 150 V for 1 h. After electrophoresis, the reaction products were visualized by staining in a 0.1% toluidine blue solution. The resulting image was used to obtain values for the pre-tRNA^{Tyr} processing activity with various incubation times. The cleavage efficiency was calculated as follows: the quantity of (matured tRNA^{Tyr} + leader fragment)/the quantity of

Fig. 2 Sensorgrams of real-time analyses by Biacore. (A) A target oligonucleotide termed Ta-SL3 containing the SL3 sequence (red) was immobilized via biotinylated oligonucleotide complementary to the nucleotide sequence at the 3'-end of Ta-SL3 on the SA sensor chip. (B) The interaction of the *Pho*Pop5–*Pho*Rpp30 complex with Ta-SL3 was analysed, as described in Materials and Methods. The *Pho*Pop5–*Pho*Rpp30 complex was injected sequentially at increasing concentrations 4.8, 24, 120, 600 nM and 3 μ M. k_{on} and k_{off} for the interaction of the *Pho*Pop5–*Pho*Rpp30 complex with Ta-SL3 were obtained by fitting sensorgrams to a Langmuir binding model shown by broken lines in red. (C, D) Sensorgrams of the interaction of *Pho*Pop5 and *Pho*Rpp30 with Ta-SL3, respectively. The proteins were injected in the same manner as that described above. K_D values for the binding of *Pho*Pop5 and *Pho*Rpp30 to Ta-SL3 were calculated by non-linear regression analysis of the steady-state values using a single site model, respectively, as presented in Supplementary Figs S1A and B. The resulting parameter values are given in Table I. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

(pre-tRNA^{Tyr} + matured tRNA^{Tyr} + leader fragment), and the percentage was plotted against the incubation times.

Modelling of PhoPop5–PhoRpp30 bound PhopRNA

Comparative analysis of the RNase P RNA sequences and existing crystallographic structural information of the bacterial RNase P RNAs were combined to generate a phylogenetically supported 3-D model of *PhoP*RNA (19). The crystal structure of *PhoP*5 in complex with *PhoR*pp30 was solved previously (Protein Data Bank accession code 2CZV) (7). The two *PhoP*5 α 4 helices in the tetramer were docked onto the *PhoP*RNA model so as to interact with double-stranded structures in SL3 and SL16 using the program Coot (20). Because the model structure has not been subjected to energy minimization, the coordinates of the structure have not been deposited in Protein Data Bank. The coordinates will, however, be provided on request from the corresponding author.

Results

Binding affinities of PhoPop5 and PhoRpp30 to SL3 measured by SPR analysis

To examine the interaction of *PhoPop5* and *PhoRpp30* with *PhopRNA* quantitatively, SPR analysis with Biacore system was chosen because one of the partners is fixed so that events subsequent to the binding step may be prevented in this analysis, as described by Baltzinger *et al.* (21). We found recently that

PhoPop5 and *PhoRpp30* specifically bind to SL3 and SL16 in the C-domain of *PhopRNA* (15). It is known that residues C252-G258 connecting P16 interact with a single-strand region (residues C55-G61) to form P6 (19). It is thus likely that *PhoPop5*–*PhoRpp30* recognizes a specific structure of SL16 formed in P6. Hence, SL3 was chosen as a target RNA for SPR analysis. To this end, a target oligonucleotide termed Ta-SL3 containing the SL3 sequence prepared by *in vitro* transcription was immobilized via biotinylated oligonucleotide complementary to the nucleotide sequence at the 3'-end of Ta-SL3 on the SA sensor chip, as shown in Fig. 2A. In SPR analysis, we employed the single-cycle kinetics introduced by Karlsson *et al.* (22), in which sequential injections of proteins (analyte) at increasing concentrations without regeneration steps between each sample injection are possible in a single binding cycle. Figure 2B–D shows typical sensorgrams of the association and dissociation of the proteins with Ta-SL3, where the proteins were injected sequentially at increasing concentrations (4.8, 24, 120, 600 nM and 3 μ M). The sensorgram clearly shows that the *PhoPop5*–*PhoRpp30* complex associates with Ta-SL3 quickly and dissociates slowly, indicating a

Table I. Kinetic constants for the interaction of the proteins with Ta-SL3

Proteins	k_{on} (1/Ms)	k_{off} (1/s)	K_D (nM)	R_{max} (RU)
<i>PhoPop5-PhoRpp30</i>	2.59×10^7	5.88×10^{-2}	2.27	793.16
<i>PhoPop5</i>	ND	ND	2.41×10^3	2,145.74
<i>PhoRpp30</i>	ND	ND	55.79×10^3	55.74
Δ L43-48- <i>PhoRpp30</i>	3.68×10^6	2.40×10^{-2}	6.52	734.52
Δ 14C- <i>PhoRpp30</i>	ND	ND	1.77×10^3	1,112

ND, not determined

specific interaction with Ta-SL3 (Fig. 2B). By fitting sensorgrams to a Langmuir binding model, k_{on} and k_{off} were unambiguously obtained, and K_D was calculated to be 2.27 nM, as presented in Table I. In contrast, *PhoPop5* or *PhoRpp30* was found to dissociate from Ta-SL3 more rapidly than the complex as shown in Figs 2C and D, respectively, and hence, k_{on} and k_{off} could not be determined accurately. In particular, no or little binding was detected when *PhoRpp30* was injected over Ta-SL3 immobilized on the SA chip (Fig. 2D). K_D values for the binding of *PhoPop5* and *PhoRpp30* to Ta-SL3 were therefore calculated to be 2.41 and 55.79 μ M, respectively, by non-linear regression analysis of the steady-state values using a single-site model (Supplementary Figs S1A and B), as described in Materials and Methods. This result indicated that *PhoPop5* is primarily responsible for binding to SL3, and that the complex formation with *PhoRpp30* remarkably enhanced binding affinity by 830- and 19,200-fold compared with those for *PhoPop5* and *PhoRpp30*, respectively.

The C-terminal helix in *PhoPop5* is a crucial element for binding to SL3

We found previously that deletion of the loop (residues 43–48) between $\alpha 1$ and $\alpha 2$ in *PhoPop5* abolished its ability to homodimerize itself and resulted in heterodimerization with *PhoRpp30*, and that the reconstituted particle containing the mutant, referred to as Δ L43-48, had little pre-tRNA cleavage activity (7, 14). In addition, the reconstituted particle containing the C-terminal-truncated *PhoPop5* mutant, Δ 14C, exhibited little pre-tRNA cleavage activity (14). Next, to gain more insight into the functional implication of *PhoPop5*, the binding affinity of Δ L43-48 and Δ 14C to Ta-SL3 was evaluated in the presence of *PhoRpp30* (Supplementary Fig. S2A and B). Their binding parameters measured are shown in Table I. Δ L43-48 and *PhoRpp30* were able to interact with Ta-SL3 at a level comparable to the wild-type *PhoPop5* and *PhoRpp30* (Supplementary Fig. S2A); the K_D value was 6.52 nM. This result indicated that the heterodimer composed of Δ L43-48 and *PhoRpp30* is sufficient for specific binding to Ta-SL3. In contrast, Δ 14C and *PhoRpp30* were found to dissociate from Ta-SL3 more rapidly than the *PhoPop5-PhoRpp30* complex as shown in Supplementary Fig. S2B. Hence, the K_D value for binding of Δ 14C and *PhoRpp30* to Ta-SL3 was calculated by non-linear regression analysis of the

steady-state values using a single-site model (Supplementary Fig. S2C), as described above. The K_D value (1.77 μ M) for the binding of Δ 14C and *PhoRpp30* to Ta-SL3 was comparable to that (2.41 μ M) for *PhoPop5* to Ta-SL3 (Table I), suggesting that Δ 14C retains the ability to bind Ta-SL3 comparable to *PhoPop5*, but has no ability to complex with *PhoRpp30*. Although we found previously that the 14 C-terminal residues in *PhoPop5* are not implicated in the formation of a complex with *PhoRpp30* (Hazeyama *et al.*, unpublished results), we measured the binding potency of Δ 14C alone to Ta-SL3 to exclude this assumption. The sensorgram clearly shows that Δ 14C had no ability to bind to Ta-SL3 (Supplementary Fig. S2D). That is, Δ 14C and *PhoRpp30* were found to decrease the binding affinity to Ta-SL3 by 780-fold compared with the wild-type complex, indicating that the C-terminal helix ($\alpha 4$) in *PhoPop5* acts as an essential element for *PhoP*RNA binding.

PhoRpp30 serves as a molecular chaperone for *PhoPop5*

We found previously that *PhoP*RNA mutant Δ P3, in which stem-loop SL3 containing P3 was deleted, had little ability to bind *PhoPop5* and *PhoRpp30* (15). In this study, SPR analysis indicated that *PhoRpp30* has little ability to bind Ta-SL3 and its tetrameric formation with *PhoPop5* remarkably enhanced the binding affinity to Ta-SL3. These results suggested that *PhoRpp30* serves as a molecular chaperone in the dimeric formation of *PhoPop5* in the tetramer. To support this assumption, the quaternary structure in solution was estimated by gel filtration on Superdex S-200. As shown in Fig. 3, *PhoRpp30* and *PhoPop5-PhoRpp30* were eluted at positions corresponding to their molecular masses, around 25,000 and 70,000, respectively, whereas *PhoPop5* was eluted at an earlier position corresponding to around 50,000, even though it has a molecular mass of 14,043. This result indicated that *PhoRpp30* exists as a monomer, whereas *PhoPop5* is an oligomer in solution. The crystal structure shows the extensive hydrophobic and ionic interactions between *PhoRpp30* and *PhoPop5* (7). It is thus likely that *PhoRpp30* assists *PhoPop5* in attaining a functionally active conformation probably by shielding the hydrophobic surfaces of *PhoPop5*.

The present result suggested that *PhoRpp30* primarily acts as a molecular chaperone for formation of the active dimeric structure of *PhoPop5*. Previous Alascaning site-directed mutagenesis of amino acids conserved in the Rpp30 family indicated that mutations of Arg90, Arg107, Lys123, Arg176, Asp180 and Lys196 in *PhoRpp30* moderately reduced pre-tRNA cleavage activity (32–50%) by the reconstituted particles containing individual mutants (16). Mapping these amino acids on the heterotetrameric structure, Arg90, Lys123 and Lys196 are located far from the interface of *PhoPop5*, although Arg107, Arg176 and Asp180 appear to be involved in the interaction with *PhoPop5* (Supplementary Fig. S3A). This finding suggested that *PhoRpp30* itself appeared to be involved in the activation of *PhoP*RNA. To examine the

Fig. 3 Gel filtration chromatography of proteins. The proteins *PhoPop5* (■ green), *PhoRpp30* (● blue), and the *PhoPop5*–*PhoRpp30* complex (○ red) were loaded onto a HiLoad 16/60 Superdex 200 column (1.6 cm × 60 cm). The arrows indicate the estimated elution positions for proteins with the molecular masses of 45,000 and 25,000. The molecular mass marker proteins used were ovalbumin (45,000) and α -chymotrypsinogen (24,800). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

functional implication of *PhoRpp30* further, we prepared these mutants and reinvestigated them with respect to the involvement in pre-tRNA cleavage activity by analysing kinetic parameters (Supplementary Fig. S3B and C).

Kinetic constants for pre-tRNA cleavage activity of the reconstituted particles containing individual mutants are summarized in Table II. The k_{cat} values (0.79 – 1.26 min^{-1}) of the mutant particles except for that containing R107A were approximately the same as that (1.11 min^{-1}) of the wild type. Mutation of Arg107 to Ala resulted in a 2.4-fold decrease in k_{cat} . In contrast, the K_M values (8.16 – $14.81 \mu\text{M}$) of the mutant particles except for that of R107A were slightly increased by about 2- to 3-fold compared with that ($5.03 \mu\text{M}$) of the wild type. The resulting specificity constants (k_{cat}/K_M) of the reconstituted particles containing mutants were reduced 1.57- to 2.75-fold in comparison to that of the particle containing the wild-type *PhoRpp30*. This result indicated that *PhoRpp30* itself has little contribution to the activation of *PhopRNA*, although one cannot exclude the possibility that *PhoRpp30* is moderately involved in substrate binding.

A 3-D model of *PhoPop5* and *PhoRpp30* in complex with *PhopRNA*

The present study indicated that the C-terminal helix ($\alpha 4$) in *PhoPop5* acts as an essential element for *PhopRNA* binding. Furthermore, reconstitution experiments indicated that the tetrameric structure is essential for the activation of *PhopRNA*, and that the

Table II. Kinetic constants of the reconstituted particles containing *PhoRpp30* or its mutants

Proteins	V_{max} ($\mu\text{M}/\text{min}$)	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1}/\mu\text{M}$)
<i>PhoRpp30</i>	0.28 ± 0.05	5.03 ± 2.13	1.11	0.22
R90A	0.20 ± 0.03	10.45 ± 2.29	0.79	0.08
R107A	0.12 ± 0.03	4.99 ± 2.69	0.46	0.09
K123A	0.29 ± 0.05	8.16 ± 2.66	1.14	0.14
R176A	0.26 ± 0.07	8.37 ± 4.31	1.03	0.12
D180A	0.31 ± 0.08	14.81 ± 5.63	1.26	0.08
K196A	0.26 ± 0.06	11.01 ± 4.61	1.03	0

C-terminal helix $\alpha 4$ in *PhoPop5* plays a crucial role in the activation of *PhopRNA* (7, 14). We found further that the *PhoPop5*–*PhoRpp30* complex specifically recognizes SL3 and SL16 (15). In the *PhopRNA* model structure (19), SL3 is approximately 65 Å away from SL16 (Fig. 4A). On the other hand, the two *PhoPop5* C-terminal helices $\alpha 4$ in the tetramer, which are crucial RNA-binding elements found in this study, are similarly 65 Å away from each other (Fig. 4B). This finding, together with previous data allowed us to generate a 3-D model, in which the two *PhoPop5* C-terminal helices ($\alpha 4$) in the tetramer bind SL3 and SL16 in *PhopRNA*, as presented in Fig. 4C. In this 3-D model, the tetramer composed of two *PhoPop5* and two *PhoRpp30* binds SL3 and SL16 in *PhopRNA*. On the one hand, the crystal structure of the *PhoRpp21*–*PhoRpp29* complex reveals that *PhoRpp21* and *PhoRpp29* fold into a dimeric structure (8),

Fig. 4 A 3-D model of the *PhoPop5*–*PhoRpp30* complex bound *PhopRNA*. (A, B) The 3-D model of *PhopRNA* and the crystal structure of the *PhoPop5*–*PhoRpp30* complex, respectively. In the *PhopRNA* model structure, SL3 in violet is approximately 65 Å away from SL16 in green, and the two *PhoPop5* C-terminal helices $\alpha 4$ (red) in the tetramer are similarly 65 Å away from each other. (C) A 3-D model of the *PhoPop5*–*PhoRpp30* complex bound *PhopRNA*. *PhoPop5* and *PhoRpp30* are in cyan and brown, respectively; and the two C-terminal helices $\alpha 4$ in *PhoPop5* are highlighted in blue. Stem-loop structures SL3 and SL16 are highlighted in red. (D) Secondary structures of SL3 and SL16 containing P3 and P16 helices, respectively. Possible structural elements recognized by the *PhoPop5*–*PhoRpp30* complex are boxed in red. Figures were drawn using Pymol (<http://www.pymol.org>). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

suggesting that the stoichiometry of the *PhoPop5*–*PhoRpp30* complex with the *PhoRpp21*–*PhoRpp29* complex is 2:1. Recently, Ma *et al.* (23) described that the stoichiometry of the proteins is 1:1 in the hyperthermophilic archaea *P. furiosus* RNase P, even though *PfuPop5* and *PfuRpp30*, homologues of *PhoPop5* and *PhoRpp30*, respectively, fold into the heterotetrameric structure in solution. To address this issue, the stoichiometry of the *P. horikoshii* RNase P proteins was examined by analysing the pre-tRNA cleavage activity of the reconstituted particles containing *PhopRNA* and various amounts of the proteins. When an increasing amount of *PhoRpp21* and *PhoRpp29* was added to the reconstituted mixture containing an equimolar amount (10 pmol) of *PhopRNA* and the other three proteins (*PhoPop5*, *PhoRpp30* and *PhoRpp38*), the reconstituted particle containing

5 pmol *PhoRpp21* and *PhoRpp29* had the full pre-tRNA cleavage activity, as shown in Fig. 5. In contrast, 20 pmol *PhoPop5* and *PhoRpp30* were required to reach full pre-tRNA cleavage activity of the reconstituted mixture containing an equimolar amount (10 pmol) of *PhopRNA* and the other three proteins (*PhoRpp21*, *PhoRpp29* and *PhoRpp38*) (Fig. 5). This result indicated a 2:1 stoichiometry of the two protein pairs, *i.e.* the tetramer composed of *PhoPop5* and *PhoRpp30* interacts with *PhopRNA* in RNase P, consistent with the model structure proposed in this study.

Discussion

Reconstitution experiments suggested that *PhoPop5* and *PhoRpp30* synergistically activate *PhopRNA* (6). Structural and mutational data indicated that

Fig. 5 Analysis of the stoichiometry of the *Pyrococcus horikoshii* RNase P proteins. Various amounts of *PhoPop5* and *PhoRpp30* or *PhoRpp21* and *PhoRpp29* were incubated with the reconstituted particles containing *PhopRNA* (10 pmol) and the other three proteins (each 10 pmol) for 15 min at 75°C. The reaction products were analysed as described previously (15). Open circles (○) indicate pre-tRNA cleavage activities of the reconstituted particles containing increasing amounts of *PhoPop5* and *PhoRpp30*, whereas closed circles (●) indicate those of the particles containing increasing amounts of *PhoRpp21* and *PhoRpp29*. The experiments were carried out in triplicate, and the mean values are presented.

PhoPop5 and *PhoRpp30* fold into a tetramer in solution, the quaternary structure playing a crucial role in the activation and stabilization of *PhopRNA* (7, 14). To understand the molecular basis of the cooperative function of *PhoPop5* and *PhoRpp30*, we first characterized them with respect to binding affinity to *PhopRNA*. Previously, gel retardation assay showed that the interactions of *PhoPop5* or *PhoRpp30* with *PhopRNA* produced a smeary shift, and the protein appeared to be aggregated on *PhopRNA* at higher protein concentrations (4). Similar observations on some RNA-binding proteins have been reported (24). These phenomena have impeded quantitative evaluation of their interaction with RNA. Recently, Baltzinger *et al.* have described that SPR analysis immobilizing a target RNA on a sensor chip can avoid events subsequent to the binding step (21). Hence, we employed SPR analysis to investigate the interaction of *PhoPop5* and *PhoRpp30* with *PhopRNA* using Ta-SL3 as a target RNA (Fig. 2). In the initial step of this analysis, no specific binding of the proteins to Ta-SL3 was observed using the reconstitution buffer, 50 mM Tris-HCl (pH 7.6) containing 50 mM MgCl₂, 600 mM NH₄OAc and 60 mM NH₄Cl, as a running buffer. As it is known that the interaction between an oligonucleotide and a positively charged molecule in SPR analysis is highly dependent on salt concentrations (21), running buffer without MgCl₂ and NH₄OAc was used, as described in Materials and Methods. As a result, a specific interaction of the *PhoPop5*–*PhoRpp30* complex and *PhoPop5* with Ta-SL3 was successfully measured and their K_D values were determined (Table I and Fig. 2). The SPR analysis was, however, carried out using Ta-SL3 under binding conditions distinct from those used

for pre-tRNA cleavage assay. Therefore, we cannot correlate their K_D values with the binding affinity of *PhopRNA* by the proteins. Nevertheless, this result indicates that *PhoPop5* is primarily responsible for binding to Ta-SL3 in the tetramer, and that its complex formation with *PhoRpp30* remarkably enhances binding affinity to Ta-SL3 by 830-fold compared with that of *PhoPop5* alone.

It should be noted that the R_{max} value (2,145.74 RU) observed in the *PhoPop5* binding to Ta-SL3 is significantly higher than that (793.16 RU) of the *PhoPop5*–*PhoRpp30* complex (Table I). This observation suggests that *PhoPop5* exists in an oligomeric structure in solution, whose molecular mass may be higher than that of the *PhoPop5*–*PhoRpp30* complex. Gel filtration analysis, however, showed that *PhoPop5* was eluted at a position corresponding to around 50,000, whereas *PhoPop5*–*PhoRpp30* was at a position corresponding to its molecular mass (70,000) (Fig. 3). Although we have no appropriate explanation for the higher R_{max} value for *PhoPop5* at the moment, it could be assumed that the interaction of *PhoPop5* with RNA results in an aggregation of *PhoPop5* on the SA sensor chip.

Based on available data, we propose a mechanistic model for the activation of *PhopRNA* by *PhoPop5* and *PhoRpp30* synergistically, as presented in Fig. 6. *PhoRpp30* exists as a monomer, whereas *PhoPop5* is an oligomer in solution, and their interaction results in attaining a functional dimeric conformation of *PhoPop5* in the tetramer. The two *PhoPop5* C-terminal helices $\alpha 4$ in the tetramer bridge SL3 and SL16, and thereby stabilize an appropriate conformation of *PhopRNA*. The proposed model suggests that *PhoRpp30* functions to shield hydrophobic surfaces of *PhoPop5* so as to prevent its aggregation and assists *PhoPop5* in attaining a functionally active conformation in the tetramer. Structural analysis revealed the extensive hydrophobic and ionic interactions between *PhoPop5* and *PhoRpp30*, including that between the N-terminal segment of *PhoPop5* and residues Asp109–Asp113 in *PhoRpp30* (7). Mutation of Arg107 in *PhoRpp30* slightly decreased the k_{cat} value: mutation of Arg107 to Ala resulted in a 2.4-fold decrease in k_{cat} (Table II and Fig. S3C). Examination of the *PhoPop5*–*PhoRpp30* complex's structure reveals that the Arg107 side chain forms a salt bridge with the Asp109 side chain at the loop between $\beta 5$ and $\alpha 5$ in *PhoRpp30*. The Asp109 side chain, in turn, water-mediated hydrogen bonds with the Leu5 main chain in *PhoPop5* and thereby stabilizes the N-terminal segment of *PhoPop5* (Supplementary Fig. S4). The N-terminal β -strand ($\beta 1$) and the C-terminal strand ($\beta 4$) form an antiparallel β -sheet, which acts as a platform for the C-terminal helix $\alpha 4$ in *PhoPop5*. It is thus assumed that mutation of Arg107 caused slight perturbation of an appropriate orientation of the C-terminal helix ($\alpha 4$) of *PhoPop5* that may result in reduction of the k_{cat} value of the reconstituted particle containing R107A.

In the presented 3-D model (Fig. 4C), two *PhoPop5* C-terminal helices $\alpha 4$ in the tetramer bridge SL3 and SL16, assuming that SL3 and SL16 have a common

Fig. 6 The proposed molecular mechanism by which *PhoPop5* and *PhoRpp30* activate *PhopRNA*. *PhoRpp30* (green) exists as a monomer, whereas *PhoPop5* (blue) is an oligomer in solution. Hydrophobic interactions of *PhoRpp30* with *PhoPop5* avoid the self-oligomerization of *PhoPop5*, which results in attaining a functional dimeric conformation of *PhoPop5* in the heterotetramer. Then, the two *PhoPop5* C-terminal helices $\alpha 4$ shown in red circles in the tetramer bind the stem-loop structures (red) containing P3 and P16 helices, and thereby stabilize an appropriate conformation of *PhopRNA*. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

structural element recognized by the C-terminal helix ($\alpha 4$) in *PhoPop5*. This assumption is derived from three different observations. First, previous mutagenesis has identified nucleotides encompassing C26 to G34 as a minimum binding site for the *PhoPop5*–*PhoRpp30* complex in SL3 (15). Second, deletion of single-stranded segments, G244–U251 and U259–A261, connecting P16 and P6 had no influence on the ability to bind the *PhoPop5*–*PhoRpp30* complex, suggesting that the complex recognizes a double-stranded structure in SL16 (F. Kumagai and M. Kimura, unpublished results). Third, we found previously that *PhoPop5* and *PhoRpp30* could activate a chimeric RNase P RNA, composed of the *Escherichia coli* RNase P RNA (M1 RNA) C-domain and the *PhopRNA* S-domain (9), suggesting that a structural element in SL3 and SL16 recognized by the C-terminal helix ($\alpha 4$) in *PhoPop5* is conserved in M1 RNA. Indeed, double-stranded structures in archaeal P3 and P16 helices are highly conserved in corresponding helices in M1 RNA. By considering these results, it is conceivable that the two *PhoPop5* C-terminal helices $\alpha 4$ in the tetramer may recognize similar double-stranded structures formed by annealing G26–G28 and C34–U32 in P3 and G240–G243 and C265–G262 in P16 (Fig. 4D).

It is known that many RNA-binding proteins have modular structures and are composed of multiple repeats of RNA recognition motif (RRM) that are arranged in various ways to satisfy their diverse functional requirements (25, 26). For instance, *Saccharomyces cerevisiae* Prp24, an essential splicing factor, contains four RRM domains and cooperatively functions to anneal U6 and U4 RNAs during spliceosome assembly. Thus, RNA-binding proteins have evolved by combining RNA-binding domains in various structural arrangements that can recognize RNA with the affinity and selectivity that is required to find cognate RNAs in the cellular medium. Although *PhoPop5* folds into RRM, it is distinct from the typical RRM in that it has an insertion of α -helix ($\alpha 2$) between

$\alpha 1$ and $\beta 2$, and in addition, it has additional helices ($\alpha 4$ and $\alpha 5$) at the C-terminus, which pack against one face of the β -sheet. *PhoPop5* dimerizes through hydrogen bonding interaction from the loop between $\alpha 1$ and $\alpha 2$ helices with the aid of *PhoRpp30*, and the C-terminal helices ($\alpha 4$) thus adopted act as a binding element for the double-stranded structure. Hence, archaeal Pop5, unlike most RNA-binding proteins, may fulfil a functional requirement by acquiring additional structural elements that facilitate the bridging of SL3 and SL16 in *PhopRNA*.

Interestingly, the fourth C-terminal RRM domain in Prp24, like *PhoPop5*, has two additional flanking α -helices that form a large electropositive surface. It has been described that the two helices are involved in destabilization of the U6 internal stem loop, a stable helix that must be unwound during U4/U6 assembly (27). Recently, we found that *PhoPop5* significantly enhanced both RNA annealing and strand displacement and thus, may assist *PhopRNA* in achieving a functionally active conformation by RNA chaperone activity (28). Deletion of the C-terminal helix $\alpha 4$ in *PhoPop5* significantly reduced not only pre-tRNA cleavage activity of the reconstituted particle but also abolished its RNA annealing and strand displacement activities (14). Thus, the C-terminal helices in the tetramer, besides bridging SL3 and SL16, may be involved in correct double-stranded formation of P3 and P16 helices as an RNA chaperone.

In contrast to archaeal RNase P RNA, eubacterial RNase P RNA itself can hydrolyse pre-tRNA *in vitro* in the presence of a high concentration of Mg^{2+} (1). The bacterial A-type RNase P RNAs have helical stems P13, P14 and P18, which are absent in the archaeal RNase P RNAs (Fig. 7) (29, 30). It is known that tetraloop–helix interactions between P8 and P14 and P8 and P18 in the bacterial A-type RNase P RNA position the two domains (C- and S-domains) correctly to permit catalysis. A shortened RNA and an increase in the number of proteins in

Fig. 7 Structural comparison of the bacterial RNase P RNA with *PhoP*RNA in complex with *PhoPop5* and *PhoRpp30*. The bacterial A-type RNase P RNA in *Thermotoga maritima* is made up of two layers; the large layer of the structure contains most of the universally conserved regions, whereas the second layer comprises helical stems P13, P14 and P18, which are absent in the archaeal RNase P RNAs (29, 30). It is assumed that the tetraloop–helix interaction between P8 (blue) and P18 (red) in the bacterial A-type RNase P RNA is replaced by the cross-linking of SL3 and SL16 by the *PhoPop5*–*PhoRpp30* complex in *PhoP*RNA. The C- and S-domains in the bacterial A-type RNase P RNA and *PhoP*RNA are in grey and gold, respectively. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

archaeal RNase Ps suggest that some structural roles of eubacterial RNase P RNA might be delegated to the proteins in archaeal RNase P. On the basis of the model of *PhoP*RNA in complex with *PhoPop5*–*PhoRpp30*, it is likely that the tetraloop–helix interaction between P8 and P18 in the bacterial A-type RNase P RNA is replaced by the cross-linking of SL3 and SL16 by the *PhoPop5*–*PhoRpp30* complex in archaeal RNase P RNAs (Fig. 7). The presented model may help guide further investigations not only into the mechanism of the *PhoPop5*–*PhoRpp30* complex-mediated activation of *PhoP*RNA but also into the molecular evolution of RNase Ps in the three phylogenetic domains of life.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

We are grateful to M. Kifusa and M. Miyanoshita for their initial experiments for analysing the stoichiometry of the archaeal RNase P proteins.

Funding

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (no. 22380062 to M.K.).

Conflict of Interest

None declared.

References

1. Kirsebom, L.A. and Trobro, S. (2009) RNase P RNA-mediated cleavage. *IUBMB Life* **61**, 189–200
2. Esakova, O. and Krasilnikov, A.S. (2010) Of proteins and RNA: the RNase P/MRP family. *RNA* **16**, 1725–1747
3. Jarrous, N. and Gopalan, V. (2010) Archaeal/Eukaryal RNase P: subunits, functions and RNA diversification. *Nucleic Acids Res.* **38**, 7885–7894
4. Kouzuma, Y., Mizoguchi, M., Takagi, H., Fukuhara, H., Tsukamoto, M., Numata, T., and Kimura, M. (2003) Reconstitution of archaeal ribonuclease P from RNA and four protein components. *Biochem. Biophys. Res. Commun.* **306**, 666–673
5. Fukuhara, H., Kifusa, M., Watanabe, M., Terada, A., Honda, T., Numata, T., Kakuta, Y., and Kimura, M. (2006) A fifth protein subunit Ph1496p elevates the optimum temperature for ribonuclease activity from *Pyrococcus horikoshii* OT3. *Biochem. Biophys. Res. Commun.* **343**, 956–964
6. Terada, A., Honda, T., Fukuhara, H., Hada, K., and Kimura, M. (2006) Characterization of the archaeal ribonuclease P proteins from *Pyrococcus horikoshii* OT3. *J. Biochem.* **140**, 293–298
7. Kawano, S., Nakashima, T., Kakuta, Y., Tanaka, I., and Kimura, M. (2006) Crystal structure of protein Ph1481p in complex with protein Ph1877p of archaeal RNase P from *Pyrococcus horikoshii* OT3: implication of dimer formation of the holoenzyme. *J. Mol. Biol.* **357**, 583–591
8. Honda, T., Kakuta, Y., Kimura, K., Saho, J., and Kimura, M. (2008) Structure of an archaeal homolog of the human protein complex Rpp21–Rpp29 that is a key core component for the assembly of active ribonuclease P. *J. Mol. Biol.* **384**, 652–662

9. Honda, T., Hara, T., Nan, J., Zhang, X., and Kimura, M. (2010) Archaeal homologs of human RNase P protein pairs Pop5 with Rpp30 and Rpp21 with Rpp29 work on distinct functional domains of the RNA subunit. *Biosci. Biotechnol. Biochem.* **74**, 266–273
10. Tsai, H.Y., Pulkunat, D.K., Woznick, W.K., and Gopalan, V. (2006) Functional reconstitution and characterization of *Pyrococcus furiosus* RNase P. *Proc. Natl. Acad. Sci. USA* **103**, 16147–16152
11. Pulkunat, D.K. and Gopalan, V. (2008) Studies on *Methanocaldococcus jannaschii* RNase P reveal insights into the roles of RNA and protein cofactors in RNase P catalysis. *Nucleic Acids Res.* **36**, 4172–4180
12. Cho, I.M., Lai, L.B., Susanti, D., Mukhopadhyay, B., and Gopalan, V. (2010) Ribosomal protein L7Ae is a subunit of archaeal RNase P. *Proc. Natl. Acad. Sci. USA* **107**, 14573–14578
13. Chen, W.Y., Pulkunat, D.K., Cho, I.M., Tsai, H.Y., and Gopalan, V. (2010) Dissecting functional cooperation among protein subunits in archaeal RNase P, a catalytic ribonucleoprotein complex. *Nucleic Acids Res.* **38**, 8316–8327
14. Hazeyama, K., Ishihara, M., Ueda, T., Nishimoto, E., Nakashima, T., Kakuta, Y., and Kimura, M. (2013) Extra-structural elements in the RNA recognition motif in archaeal Pop5 play a crucial role in the activation of RNase P RNA from *Pyrococcus horikoshii* OT3. *Biochem. Biophys. Res. Commun.* **440**, 594–598
15. Ueda, T., Yamaguchi, H., Miyano, M., Nakashima, T., Kakuta, Y., and Kimura, M. (2014) Characterization of the peripheral structures of archaeal RNase P RNA from *Pyrococcus horikoshii* OT3. *J. Biochem.* **155**, 25–33
16. Takagi, H., Watanabe, M., Kakuta, Y., Kamachi, R., Numata, T., Tanaka, I., and Kimura, M. (2004) Crystal structure of the ribonuclease P protein Ph1877p from hyperthermophilic archaeon *Pyrococcus horikoshii* OT3. *Biochem. Biophys. Res. Commun.* **319**, 787–794
17. Myska, D.G. (1999) Improving biosensor analysis. *J. Mol. Recognit.* **12**, 279–284
18. Palau, W. and Primo, C.D. (2012) Single-cycle kinetic analysis of ternary DNA complexes by surface plasmon resonance on a decaying surface. *Biochimie* **94**, 1891–1899
19. Zwieb, C., Nakao, Y., Nakashima, T., Takagi, H., Goda, S., Andersen, E.S., Kakuta, Y., and Kimura, M. (2011) Structural modeling of RNase P RNA of the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3. *Biochem. Biophys. Res. Commun.* **414**, 517–522
20. Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132
21. Baltzinger, M., Sharma, K.K., Mely, Y., and Altschuh, D. (2013) Dissecting the oligonucleotide binding properties of a disordered chaperone protein using surface plasmon resonance. *Nucleic Acids Res.* **41**, 10414–10425
22. Karlsson, R., Katsamba, P.S., Nordin, H., Pol, E., and Myska, D.G. (2006) Analyzing a kinetic titration series using affinity biosensors. *Anal. Biochem.* **349**, 136–147
23. Ma, X., Lai, L.B., Lai, S.M., Tanimoto, A., Foster, M.P., Wysocki, V.H., and Gopalan, V. (2014) Uncovering stoichiometry of *Pyrococcus furiosus* RNase P, a multisubunit catalytic ribonucleoprotein complex, by surface-induced dissociation and ion mobility mass spectrometry. *Angew. Chem. Int. Ed. Engl.* **53**, 11483–11487
24. Doetsch, M., Schroeder, R., and Furtig, B. (2011) Transient RNA-protein interactions in RNA folding. *FEBS J.* **278**, 1634–1642
25. Lunde, B.M., Moore, C., and Varani, G. (2007) RNA-binding proteins: modular design for efficient function. *Nat. Rev. Mol. Cell Biol.* **8**, 479–490
26. Martin-Tomasz, S., Richie, A.C., Clos, L.J. II, Brow, D.A., and Butcher, S.E. (2011) A novel occluded RNA recognition motif in Prp24 unwinds the U6 RNA internal stem loop. *Nucleic Acids Res.* **39**, 7837–7847
27. Wahl, M.C., Will, C.L., and Luhrmann, R. (2009) The spliceosome: design principles of a dynamic RNP machine. *Cell* **136**, 701–718
28. Ishihara, M., Nishimoto, E., Yamashita, S., Kakuta, Y., and Kimura, M. (2012) A distinct binding mode of archaeal Ribonuclease P proteins to the RNA. *Biosci. Biotechnol. Biochem.* **76**, 2335–2337
29. Torres-Larios, A., Swinger, K.K., Krasilnikov, A.S., Pan, T., and Mondragon, A. (2005) Crystal structure of the RNA component of bacterial ribonuclease P. *Nature* **437**, 584–587
30. Reiter, N.J., Osterman, A., Torres-Larios, A., Swinger, K.K., Pan, T., and Mondragon, A. (2010) Structure of a bacterial ribonuclease P holoenzyme in complex with tRNA. *Nature* **468**, 784–789