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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

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ABSTRACT

Ribonuclease P (RNase P) is a ribonucleoprotein complex essential for the processing of 5′ leader sequences of precursor tRNAs (pre-tRNA). *Pho*Pop5 is an archaeal homolog of human RNase P protein hPop5 involved in the activation of RNase P RNA (*Pho*pRNA) in the hyperthermophilic archaeon *Pyrococcus horikoshii*, probably by promoting RNA annealing (AN) and RNA strand displacement (SD). Although *Pho*Pop5 folds into the RNA recognition motif (RRM), it is distinct from the typical RRM in that it has an insertion of α-helix (α2) between α1 and β2. Biochemical and structural data have shown that the dimerization of *Pho*Pop5 through the loop between α1 and α2 is required for the activation of *Pho*pRNA. In addition, *Pho*Pop5 has additional helices (α4 and α5) at the C-terminus, which pack against one face of the β-sheet. In this study, we examined the contribution of the C-terminal helices to the activation of *Pho*pRNA using mutation analyses. Reconstitution experiments and fluorescence resonance energy transfer (FRET)-based assays indicated that deletion of the C-terminal helices α4 and α5 significantly influenced on the pre-tRNA cleavage activity and abolished AN and SD activities, while that of α5 had little effect on these activities. Moreover, the FRET assay showed that deletion of the loop between α1 and α2 had no influence on the AN and SD activity. Further mutational analyses suggested that basic residues at α4 are involved in interaction with *Pho*pRNA, while hydrophobic residues at α4 participate in interaction with hydrophobic residues at the β-sheet, thereby stabilizing an appropriate orientation of the helix α4. Together, these results indicate that extra-structural elements in the RRM in *Pho*Pop5 play a crucial role in the activation of *Pho*pRNA.

1. Introduction

Ribonuclease P (RNase P) is a ribonucleoprotein (RNP) that catalyzes the processing of 5′ leader sequences from tRNA precursors (pre-tRNA) and other noncoding RNAs in all living cells [1,2]. Although the functionality of RNase P remains almost the same from bacteria to humans, the chemical composition of this enzyme differs in the three phylogenetic domains of life. Eubacterial RNase P is composed of a catalytic RNA and a single protein subunit, and in the presence of a high concentration of Mg^{2+}, the eubacterial RNase P RNA itself can hydrolyze pre-tRNA *in vitro* [3]. In contrast, archaeal and eukaryotic RNase Ps comprise a single RNA moiety and multiple proteins: 4 or 5 for archaeal RNase Ps and as many as 10 for eukaryotic RNase Ps [4]. Although the RNA components in archaea and eukaryotes alone have little catalytic activity *in vitro* [5,6], they function in cooperation with protein subunits in substrate recognition and catalysis. Hence, archaeal and eukaryotic RNase Ps may serve as a model RNP for studying how a functional RNA can be activated by protein cofactors and how the RNP enzymes catalyze biological processes.

We earlier found that RNase P RNA (*Pho*pRNA) and five proteins in the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 reconstituted RNase P activity that exhibits enzymatic properties like those of the authentic enzyme [7,8]. The *P. horikoshii* RNase P proteins were designated *Pho*Pop5, *Pho*Rpp38, *Pho*Rpp21,

Abbreviations: AN, RNA annealing; CD, circular dichroism; FRET, fluorescence resonance energy transfer; *Pho*pRNA, ribonuclease P RNA from *P. horikoshii*; RNase P, ribonuclease P; pre-tRNA, precursor tRNA; SD, strand displacement.

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*Pho*Rpp29, and *Pho*Rpp30, according to their sequence homology with the human RNase P proteins hPop5, Rpp21, Rpp29, Rpp30, and Rpp38, respectively [9]. Biochemical and structural studies revealed that *Pho*Pop5 and *Pho*Rpp21 form a complex with *Pho*Rpp30 and *Pho*Rpp29, and the resulting complexes, *Pho*Pop5-*Pho*Rpp30 and *Pho*Rpp21-*Pho*Rpp29, are involved in activation of the C- and S-domains, respectively [10–12]. Recently, fluorescence resonance energy transfer (FRET)-based assays indicated that *Pho*Pop5, *Pho*Rpp21, *Pho*Rpp29, and *Pho*Rpp30 can promote RNA annealing (AN) and strand displacement (SD), while *Pho*Rpp38 has no influence on FRET [13]. Hence, the four proteins may assist *Pho*pRNA in attaining a functionally active conformation by promoting AN and SD.

*Pho*Pop5 consists of a five-stranded antiparallel β-sheet and five helices, which fold in a way that is topologically similar to the RNA recognition motif (RRM) [10]. *Pho*Pop5 is, however, distinct from the typical RRM in that it has an insertion of α-helix (α2) between α1 and β2. In addition, it has additional helices (α4 and α5) at the C-terminus, which pack against one face of the β-sheet and form an electropositive patch suitable for RNA binding [10]. It was previously shown that Glu44 and Glu48 at the loop between α1 and α2 are involved in the dimerization of *Pho*Pop5, and that this dimerization is required for the pre-tRNA cleavage activity [10]. In this study, to evaluate the involvement of the C-terminal extension in the functional activity of *Pho*Pop5, we prepared two mutants, Δ6C and Δ14C, in which the 6 and 14 C-terminal amino acids were deleted, respectively, and characterized them with respect to pre-tRNA cleavage activity and to the promoting activity of AN and SD. Truncation of 14 residues significantly reduced the pre-tRNA cleavage activity and abolished both AN and SD activities, while that of 6 residues had little effect on these activities. Moreover, it was found that deletion of the loop between α1 and α2 had no influence of FRET efficiency. Further mutational analyses indicated that positively charged residues, Lys108 and Lys112, at α4 are fully involved in interaction with *Pho*pRNA, while hydrophobic residues, such as Phe113 and Leu114, are involved in hydrophobic interaction with the β-sheet, thereby stabilizing an appropriate orientation of the helix α4. The present study shows that extra-structural elements in the RRM in *Pho*Pop5 play a crucial role in the activation of *Pho*pRNA; that is, the C-terminal extension in the dimerized *Pho*Pop5 through the loop between α1 and α2 is essential for the activation of *Pho*pRNA by promoting AN and SD.

2. Materials and methods

2.1. Materials

Five RNase P proteins (*Pho*Pop5, *Pho*Rpp21, *Pho*Rpp29, *Pho*Rpp30, and *Pho*Rpp38), *Pho*pRNA, and pre-tRNA^{Tyr} in *P. horikoshii* were prepared as described previously [7,8]. The deletion mutant ΔL43–48, in which the residues 43–48 including Glu44 and Glu48 were deleted, was prepared as described previously [10]. Restriction enzymes were purchased from MBI Fermentas (Ontario, Canada). Oligonucleotides were purchased from Sigma–Aldrich (St. Louis, MO). Two fluorescence-labeled synthetic oligoribonucleotides (Cy3-21R− and Cy5-21R+) were purchased from Operon Biotechnologies (Tokyo, Japan). Ex Taq DNA polymerase and the DNA ligation kit were purchased from Takara Bio (Shiga, Japan). All other chemicals were of analytical grade for biochemical use.

2.2. Preparation of mutant proteins

Deleted mutations and site-directed mutagenesis were produced by oligonucleotide-based mutagenesis. Mutations were identified by DNA sequence determination; the entire sequence of each mutant gene was determined to rule out that any additional mutations had arisen during the mutagenesis reaction steps. The resulting mutants were purified using the same protocol as used for the wild-type *Pho*Pop5 [10].

2.3. Far-ultraviolet circular dichroism (CD)

CD spectra in the far-ultraviolet range, 200–250 nm, were recorded at room temperature on a Jasco J-720 spectropolarimeter. Proteins were dissolved to a final concentration of 100 μg/mL in 50 mM Tris–HCl, pH 7.5, containing 200 mM NaCl. Signal averaging during the accumulation of four scans was done automatically.

2.4. Assay for pre-tRNA cleavage activity

The RNase P activity for the reconstituted particles containing *Pho*Pop5 or its mutants was analyzed, principally as described previously [14]. The reaction mixtures were incubated in the reconstitution buffer, 50 mM Tris–HCl (pH 7.6) containing 50 mM $MgCl_2$, 600 mM NH_4OAc, 60 mM NH_4Cl, *Pho*pRNA or its mutants (20 pmol), the five proteins (each 20 pmol), and pre-tRNA^{Tyr} (2.5 μg), at 75 °C for specified periods. 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 (pre-tRNA^{Tyr} + matured tRNA^{Tyr} + leader fragment), and the percentage was plotted against the incubation times.

2.5. FRET-based assay

FRET assays were principally performed as described previously [15], with some modifications [13]. Two fluorescence-labeled RNAs, Cy3-21R− (Cy3 – 5′ – ACUGCUAGAGAUUUUCCACAU-3′) and Cy5-21R+ (Cy5 – 5′ – AUGUGGAAAAUCUCUAGCAGU-3′), were obtained from Operon Biotechnologies (Tokyo). A nonfluorescence-labeled competitor RNA (21R−) with a sequence identical to Cy3-21R− was produced by *in vitro* transcription with T7 RNA polymerase using a corresponding synthetic DNA oligonucleotide as template. The resulting RNA was purified by ion-exchange column chromatography on a HiTrap DEAE Sepharose FF column, as described by Easton et al. [16]. We monitored FRET from the donor Cy3 to the acceptor Cy5 in 50 mM Tris–HCl (pH 7.5) containing 3 mM $MgCl_2$ and 1 mM DTT at 30 °C by measuring the fluorescence emission at 590 nm of Cy3 with excitation at 535 nm every 2 s using a fluorescence spectrophotometer (F-3010, Hitachi, Tokyo). In the first reaction (phase I), Cy3-21R− and Cy5-21R+ were annealed in a cell (1 mL). Annealing was started by mixing 300 μL of 100 nM Cy3-21R− with an equal volume of 100 nM Cy5-21R+ in the absence or presence of 200 μL of the protein (1 μM). The reaction was allowed to proceed for 1200 s. For the second reaction (phase II), 200 μL of 100 nM 21R− was added, the mixture was agitated vigorously, and measurements were continued for another 1200 s. The energy transfer efficiency (E) was calculated as follows: $E = 1 - F_{AD}/F_D$, where F_D and F_{AD} are fluorescence intensities of the donor (Cy3-21R−) in the absence and presence of the acceptor (Cy5-21R+), respectively [17]. Furthermore, the normalized energy transfer efficiency (NE) was calculated as $NE = E_t/E_{1200}$ in phase I and $NE = (E_t - E_{2400})/(E_{1200} - E_{2400})$, where E_t is the E at each time, and E_{1200} and E_{2400} are the values of E at 20 and 40 min, respectively.