Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effects of external molecular factors on adaptation of bacterial RNase P ribozymes to thermophilic conditions

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ARTICLE INFO

Article history:
Received 9 December 2019
Received in revised form
12 December 2019
Accepted 12 December 2019
Available online xxx

Keywords:
LUCA
Ribozyme
Ribonuclease P
Thermostability
tRNA processing

ABSTRACT

Ribonuclease P (RNase P) is an RNA processing enzyme essential for production of functional tRNAs. Bacterial RNase P is a ribozyme, i.e., an RNA-based enzyme, which functions in all bacteria including those growing at high temperatures (≥55 °C). We examined three bacterial RNase P ribozymes, one from a mesophilic bacterium and two from thermophilic bacteria, to understand the factor(s) providing efficient catalytic ability under conditions of high temperature. Thermophilic RNase P ribozymes show structural adaptations to allow correct folding at high temperature. The presence of a molecular crowder significantly enhanced the catalytic efficiency of thermophilic RNase P ribozyme reactions at 55 °C, while it modestly reduced the upper limit of the reaction temperature.

1. Introduction

Ribonuclease P (RNase P) is a class of RNA processing enzymes, the primary role of which is to remove 5′ leader sequences from precursor tRNAs (Fig. 1A) [1]. Bacterial RNase P enzymes are commonly composed of one large RNA (typically *ca.* 300–400 nucleotides) and one small basic protein (*ca.* 120 amino acids). Bacterial RNase P is classed as a ribozyme because its catalytic ability originates from its RNA component, which can promote the site-specific cleavage of pre-tRNAs *in vitro* without the protein component [1,2]. Bacterial RNase P ribozymes have a modular architecture consisting of two structural domains [1,3,4]: the C-domain (catalytic domain) containing elements essential for catalysis, and the S-domain (specificity domain) that plays an important role in the specific recognition of pre-tRNA substrates (Fig. 1B) [5,6].

As cleavage of the 5′ leader sequence is an essential step in production of functional tRNAs, RNase P ribozymes have to function in all bacterial species growing under various environmental conditions, including various growth temperatures. The RNase P ribozyme is also considered to have emerged at an early stage in the molecular evolution of life and to have functioned in the last universal common ancestor (LUCA) [7], which was proposed to have lived in hydrothermal vents [8]. Bacteria in several phylums, such as *Aquificales, Firmicutes, Thermotogae*, and *Deinococcus–Thermus*, are known to grow under conditions of high temperature (65 °C–90 °C). They also contain RNase P ribozymes [9–12], which need to be active at their growth temperature. The RNA components of these ribozymes may have adaptations for the high temperature growth conditions, and may require the assistance of external factors including their protein components, other intracellular macromolecules that provide molecular crowding conditions, and inorganic/organic cations other than Mg^{2+}. Thermophilic RNase P ribozymes from thermophilic bacteria are also interesting from the viewpoint of the RNA world hypothesis because it has been proposed that the RNA world may have emerged at hydrothermal vents [13,14].

This study was performed to investigate the thermophilic properties of two bacterial RNase P ribozymes from *Thermus thermophilus* (growing at 75 °C and abbreviated as Tth in this study) and *Thermotoga maritima* (growing at 80 °C and abbreviated as Tm in this study) to understand their folding and catalytic properties [9,10]. We also analyzed the *Escherichia coli* RNase P ribozyme

Abbreviations: Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; K10, deca-lysine; LUCA, last universal common ancestor; PEG, polyethylene glycol; Tm, *Thermotoga maritima*; Tth, *Thermus thermophilus*.

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https://doi.org/10.1016/j.bbrc.2019.12.056
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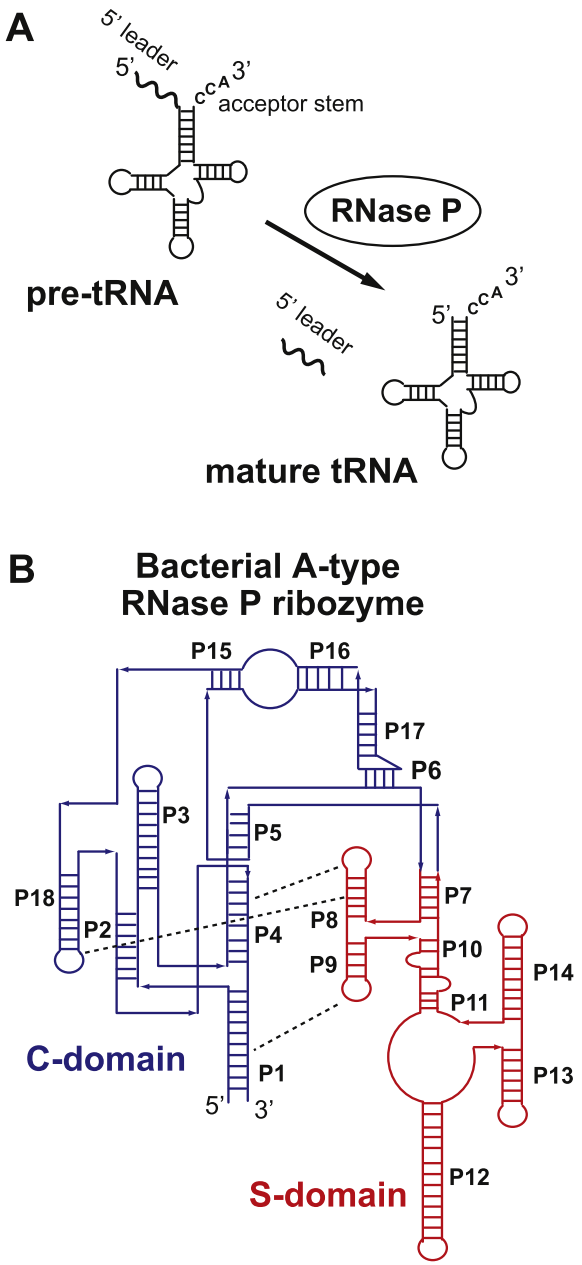

Fig. 1. Reaction catalyzed by RNase P ribozymes sharing a common secondary structure.
A) Site-specific cleavage of pre-tRNA catalyzed by RNase P enzymes.
B) Consensus secondary structure of bacterial A-type RNase P ribozyme, which consists of the C
-domain (shown in blue) and S-domain (shown in red). The two domains are covalently connected at the P5 and P7 regions. Broken lines indicate tertiary structures participating in assembly between the C-domain and S-domain. The diagram was drawn following the style of references [5,6]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(abbreviated as Ec in this study) [2]. The three ribozymes belonging to A-type (ancestral type) of bacterial RNase P ribozymes (Fig. 1B) that have similar secondary structures (Fig. S1) [1]. We performed comparative analysis of their folding and catalytic properties under mesophilic (37 °C) and thermophilic (≥55 °C) conditions in the absence or presence of external factors serving as model molecules to mimic intracellular components.

2. Materials and methods

2.1. RNA synthesis

DNA fragments encoding the sequences of each ribozyme and human tyrosyl pre-tRNA (pre-tRNA^{Tyr}) were prepared by PCR using plasmids encoding the corresponding DNA sequences as templates. Construction of plasmid DNAs bearing the sequences of Ec ribozyme, Tth ribozyme, and human pre-tRNA^{Tyr} were described previously [15,16]. A plasmid DNA bearing the sequence of the Tm ribozyme was purchased from an artificial gene synthesis service (Fasmac, Kanagawa, Japan). For PCR amplification of each template DNA for transcription, the sense primer had the T7 promoter at its 5′ end. Template DNAs for Tth glycyl pre-tRNA (pre-tRNA^{Gly}) and *Bacillus subtilis* (abbreviated as Bs in this study) aspartyl pre-tRNA (pre-tRNA^{Asp}) were prepared by primer extension with pairs of long oligonucleotides. Transcription reactions were performed for 4.5 h at 37 °C. RNAs were purified on 9% polyacrylamide gels containing 8 M urea. The 3′ ends of the ribozyme RNAs and pre-tRNAs were labeled with BODIPY fluorophore according to the published protocol [17].

2.2. Native polyacrylamide gel electrophoresis (Native PAGE)

An aqueous solution containing RNase P RNA or pre-tRNA labeled with BODIPY (final concentration: 1.0 μM) was heated at 85 °C for 5 min. To the RNA solution was added 10× concentrated folding buffer (final concentrations: 50 mM Tris-acetate, pH 7.5, and given concentration of $Mg(OAc)_2$). The resulting solution was incubated at the target temperature for 30 min and then at 4 °C for an additional 30 min. After addition of 6× loading buffer containing 50% glycerol and 0.1% xylene cyanol, the samples were loaded onto a 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide) containing 50 mM Tris-acetate (pH 7.5) and a given concentration of $Mg(OAc)_2$. Electrophoresis was carried out at 4 °C, 200 V for the initial 5 min, followed by 75 V for 5 h. The resulting gels were visualized and analyzed using a Pharos FX fluoroimager (Bio-Rad, Hercules, CA).

2.3. Pre-tRNA cleavage reaction with excess amounts of ribozyme

An aqueous RNA solution containing RNase P ribozyme (final concentration: 1.0 μM) and a similar solution containing pre-tRNA (final concentration: 0.5 μM) were prepared in separate tubes. The two RNA solutions were heated separately at 85 °C for 5 min and then cooled to a given temperature. After adding 5× concentrated reaction buffer (final concentrations: 50 mM Tris-acetate or Tris-HCl, pH 7.5 at the given temperature, 50 mM $Mg(OAc)_2$ or $MgCl_2$) and additive (KCl, PEG 8000 and/or deca-lysine (K10) peptide), two RNA solutions were further incubated separately for 30 min at the given temperature. In the case of reaction under high (≥64 °C) temperature, the preincubation step was altered to 55 °C for 25 min followed by an additional 5 min at the given temperature. The pre-tRNA cleavage reaction was initiated by mixing the two RNA solutions. Aliquots were taken at given time points and treated with 1.0 volume of stop solution 75% formamide, 0.1% xylene cyanol, and 100 mM EDTA. The reaction mixtures were separated by 9% polyacrylamide gels containing 8 M urea. The substrate and reaction products were analyzed using a Pharos FX fluoroimager. Assays were repeated at least twice, and the mean values are shown in the figures, with the minimum and maximum values indicated by error bars.

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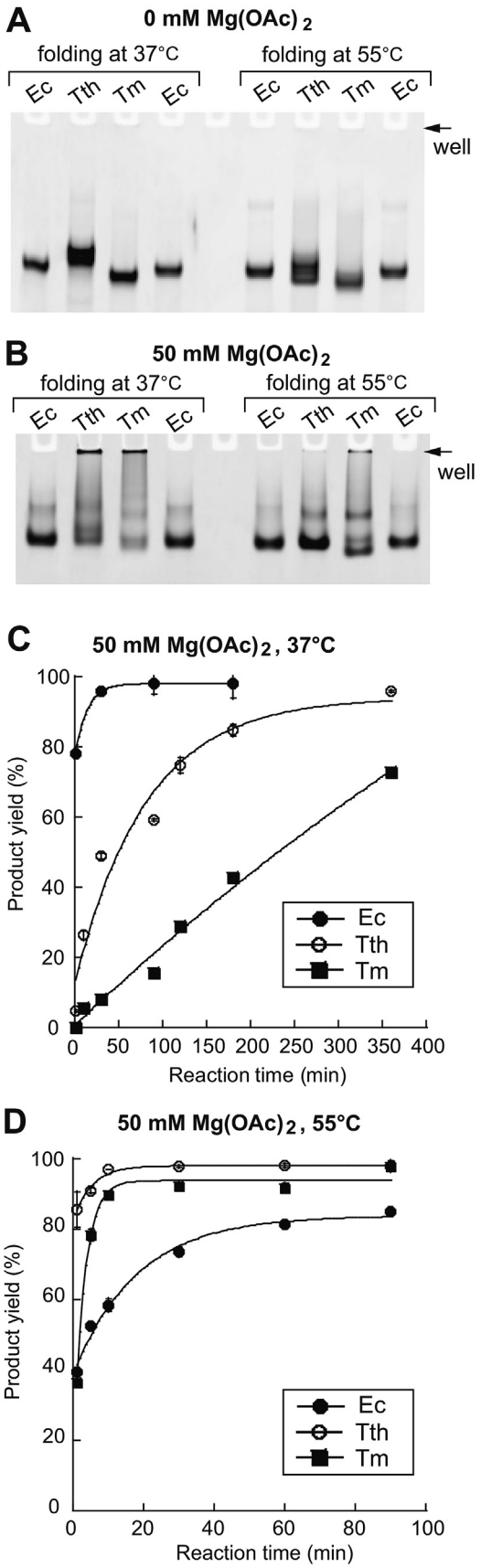

Fig. 2. Effects of folding temperature on tertiary structure and catalytic ability of the three RNase P ribozymes.
A) Native PAGE of the three RNase P ribozymes prefolded without Mg^{2+}.
B) Native PAGE of the three RNase P ribozymes prefolded with 50 mM Mg^{2+}.
(C, D) Site-specific cleavage reactions of pre-tRNA catalyzed by RNase P ribozymes at 37 °C (C) or 55 °C (D). Prefolding and reactions were performed at 37 °C (C) or 55 °C (D) with 50 mM Tris-acetate (pH 7.5) and 50 mM $Mg(OAc)_2$ in the presence of 0.5 μM human pre-$tRNA^{Tyr}$ and 1.0 μM RNase P RNA.

2.4. Pre-tRNA cleavage reaction with catalytic amounts of ribozyme

Pre-tRNA cleavage reactions were carried out with excess amounts of pre-tRNA and catalytic amounts of RNase P ribozyme by applying the activity assay protocol for conditions of ribozyme excess, steps that included RNA folding, ribozyme-catalyzed pre-tRNA cleavage reaction, denaturing polyacrylamide gel electrophoresis (PAGE) of the reaction mixture, and product analysis using the fluoroimager. To determine the K_m for substrate and V_{max} in the multiple turnover reaction, pre-tRNA cleavage reactions were performed with 1.0 nM RNase P ribozyme and an excess amount of the pre-tRNA (50 nM–2000 nM). The resulting initial velocities were then fitted using the Michaelis–Menten equation: $V = (V_{max}[\text{substrate}])/(K_m + [\text{substrate}])$.

3. Results

3.1. Effects of temperature on folding of bacterial A-type RNase P ribozymes

We first examined the effects of solution temperature on the folding of the three ribozymes. Folding states of the ribozyme RNAs were analyzed by native PAGE at 4 °C, under conditions where the RNA structures formed in the folding step can be preserved during electrophoresis. In the absence of $Mg(OAc)_2$ where ribozyme RNAs predominantly formed their secondary structures, the Ec and Tm ribozymes showed single bands the relative mobilities of which seemed to be weakly affected by folding temperature (Fig. 2A). The Tth ribozyme, however, formed multiple bands and their relative mobilities were altered by the folding temperature (Fig. 2A). These observations suggested that the Tth ribozyme may form heterogeneous secondary structures without Mg^{2+}.

In the presence of 50 mM $Mg(OAc)_2$, the Ec ribozyme RNA showed a predominant fast-migrating band, while a minor population showed a broad band with low mobility (Fig. 2B). The major band seemed insensitive to folding temperature between 37 °C and 55 °C (Fig. 2B and S2A). In the remaining two ribozymes, however, the dominant population remained in the sample well when folded at 37 °C (Fig. 2B left), suggesting that they had folding problems resulting in aggregation at 37 °C [12]. This folding problem was, however, largely solved at 55 °C, at which temperature the Tth ribozyme formed a dominant fast-migrating band the mobility of which was closely similar to that of the Ec ribozyme (Fig. 2B right). The apparent aggregated state of the Tm ribozyme at 37 °C was also solved at 55 °C to yield fast-migrating bands, one of which had faster mobility than the Ec and Tth ribozymes (Fig. 2B right).

To investigate whether the fast-migrating band of each ribozyme corresponded to the catalytically active structure, we analyzed their catalytic activities in the presence of twofold excess amounts of ribozyme (1.0 μM) over pre-tRNA substrate (0.5 μM) (Fig. 2C–D). Under these conditions, the initial burst of the reaction (reaction within 1 min) reflected the relative amount of the catalytically active ribozyme. Large (78%) and moderate (39%) initial burst fractions were observed for the Ec ribozyme at 37 °C (Fig. 2C) and 55 °C (Fig. 2D), respectively. These results were consistent with the results of native PAGE, in which the Ec ribozyme RNA dominantly formed the fast-migrating band (Fig. 2B), which could correspond to the catalytically active state. In the pre-tRNA cleavage reactions with the Tth and Tm ribozymes at 37 °C, the Tth

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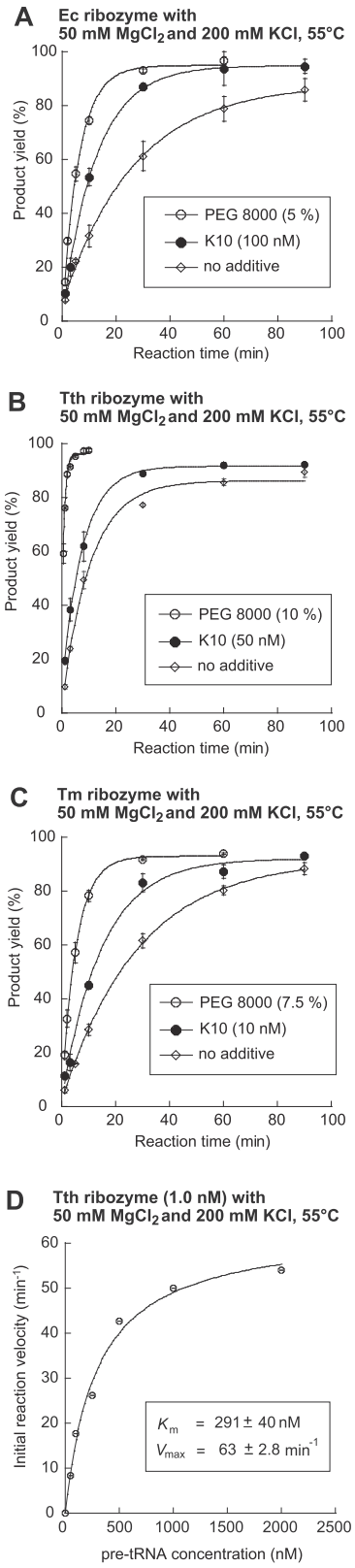

Fig. 3. Effects of PEG 8000 and K10 peptide on the three RNase P ribozymes under the conditions of substrate excess at 55 °C. In A – C, reactions were carried out with 250 nM human pre-tRNA^{Tyr} and 25 nM RNase P ribozyme. In D, reactions were carried out with 1.0 nM RNase P ribozyme. The reaction buffer consisted of 50 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, and 200 mM KCl.
A – C) Time courses of pre-tRNA cleavage reactions catalyzed by the Ec RNase P ribozyme, the Tth RNase P ribozyme (B), and the Tm RNase P ribozyme (C).
D) Michaelis–Menten plot of the initial velocities (k_{obs} values) against concentration of the substrate pre-tRNA.

ribozyme poorly showed the initial burst fraction (4.8%) and the Tm ribozyme showed no initial burst (Fig. 2C). These observations were consistent with the formation of aggregated states in the native gel (Fig. 2B). With cleavage reactions at 55 °C, however, the Tth ribozyme exhibited a marked increase of the initial burst fraction (85%) (Fig. 2D), which was even larger than that with the Ec ribozyme at 37 °C (78%) (Fig. 2C). This observation was consistent with complete resolution of the aggregated state of the Tth ribozyme when it folded at 55 °C (Fig. 2B). The considerable increase in the initial burst fraction in the reaction at 55 °C was also observed in the Tm ribozyme (36%) (Fig. 2D), aggregation of which was largely solved when it folded at 55 °C (Fig. 2B). In the folding of the Tth and Tm ribozymes, the aggregated states were also solved in a folding temperature-dependent manner (Figs. S2B and S2C). In the case of the Tth ribozyme, we also found that the correctly folded state seen at 55 °C was thermodynamically more stable than the aggregated state formed at 37 °C because the fast-migrating band formed at 55 °C remained even after additional incubation at 37 °C (Fig. S2D). We also confirmed that the reactions of Tth and Tm ribozymes folded with two temperature steps showed significant initial burst fractions (Figs. S2E and S2F).

We next investigated the effects of external factors added to the basal reaction buffer on folding and catalysis of the three RNase P ribozymes at 55 °C. We first investigated the effects of KCl because the concentration of KCl in reaction buffers often affects the catalytic ability of large ribozymes [18–20]. To determine the effects of KCl on the RNase P ribozyme reactions, we altered the source of Mg^{2+} ions from $Mg(OAc)_2$ to $MgCl_2$. This change caused no significant difference in the catalytic behavior of the three ribozymes at 37 °C and 55 °C (Fig. S3). The pre-tRNA cleavage reactions with twofold excess amounts of each RNase P ribozyme were examined in the presence of various amounts of KCl (Fig. S4). The activity of the Tth ribozyme, which was highly active even without KCl, was not negatively affected by KCl (Fig. S4B). The other two ribozymes (Ec and Tm ribozymes) showed significant increases in the initial burst fraction with increasing KCl concentration in the buffer (Figs. S4A and S4C). With 200 mM KCl, their initial burst fractions both exceeded 80% and became comparable to that with the Tth ribozyme (Figs. S4A and S4C). We used a buffer containing 50 mM $MgCl_2$ and 200 mM KCl as the standard buffer in the subsequent assays.

3.2. Activity of RNase P ribozymes under multiple turnover conditions

Based on the results of the activity assay under conditions of ribozyme excess, we next investigated the catalytic abilities of the three RNase P ribozymes to evaluate their multiple turnover ability in the presence of excess amounts of the pre-tRNA substrate. We evaluated their turnover capabilities in solution mimicking intracellular conditions. For this purpose, we added deca-lysine (K10) peptide as a model oligopeptide mimicking the protein component of bacterial RNase P because K10 peptide has been shown to enhance the catalytic activity of the Ec ribozyme [21]. We also added polyethylene glycol 8000 (PEG 8000) as a model polymer to mimic intracellular molecular crowding conditions [22].

In the presence of a 10-fold excess amount of pre-RNA (250 nM) over a given ribozyme (25 nM) in the presence of 50 mM $MgCl_2$ and 200 mM KCl, the ribozyme-catalyzed pre-tRNA cleavage reactions were performed at 55 °C (Fig. 3). The multiple turnover activities of the three ribozymes were enhanced strongly by PEG 8000 and

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modestly by K10 peptide although the optimum concentrations varied among the three ribozymes (Fig. S5). In multiple turnover reactions, the Ec and Tm ribozymes showed single exponential time courses suggesting that there was no or little initial burst fraction (Fig. 3A and C). The reaction catalyzed by the Tth ribozyme in standard buffer also showed no significant fraction (<10%) to yield the product tRNA within the initial 1 min of the reaction (Fig. 3B). In the reaction of the Tth ribozyme in the presence of 50 nM of K10 peptide, the product yield of tRNA in the initial 1 min of the reaction increased to 19% (Fig. 3B), indicating that the Tth ribozyme turned over to cleave more than one pre-tRNA within the initial 1 min. The significant turnover capability of the Tth ribozyme was observed in the presence of 10% PEG 8000 (Fig. 3B), under which conditions the product yield reached 76% in the first 1 min of the reaction. This result indicated that more than seven pre-tRNA molecules were cleaved by one ribozyme molecule in 1 min. We also examined whether PEG 8000 and K10 peptide can enhance the three RNase P ribozymes in an additive or synergistic manner. In the presence of PEG 8000 and K10 peptide, the activity of each ribozyme was close but did not exceed the activity in the presence of PEG 8000 alone (Fig. S6). These results indicate that the turnover ability of the Tth ribozyme was significantly enhanced under conditions of molecular crowding induced by 10% PEG 8000. Under multiple turnover conditions at around 37 °C, the overall rate of the RNase P ribozyme catalyzed reaction is usually limited not by hydrolytic cleavage of the phosphodiester bond but by the association and dissociation between tRNA and RNase P ribozyme.

To quantify the turnover ability of the Tth ribozyme at 55 °C with 10% PEG 8000, we determined the kinetic parameters (k_{cat} and K_m) of the reaction in the presence of 1.0 nM ribozyme and excess amounts of pre-tRNA. We fitted the initial velocity of the reaction to the Michaelis–Menten equation (Fig. 3D) and estimated K_m to be 291 nM and k_{cat} to be 63 min^{-1}. The catalytic efficiency (k_{cat}/K_m) of the Tth ribozyme was 217 $min^{-1}/\mu M$. Although buffer systems and pre-tRNA substrates were different, this k_{cat}/K_m value (217 $min^{-1}/\mu M$) is 8.7-fold higher than the previously reported value for the same ribozyme without molecular crowder at 55 °C (25 $min^{-1}/\mu M$) [23] and also 54-fold higher than that of the Ec ribozyme without molecular crowder at 37 °C (4.0 $min^{-1}/\mu M$) [24].

3.3. Upper limit of temperature in the pre-tRNA cleavage reactions by the RNase P ribozymes

We then investigated the ribozyme-catalyzed pre-tRNA cleavage reaction at temperatures higher than 55 °C. In the pre-tRNA cleavage reaction by RNase P, both pre-tRNA and the RNase P ribozyme need to form their tertiary structures correctly. As the tertiary structure of human pre-$tRNA^{Tyr}$ may not be stable at higher temperature, we used two additional substrates, i.e., Tth pre-$tRNA^{Gly}$ and Bs pre-$tRNA^{Asp}$ (Fig. S7). To examine their stability against irreversible thermal unfolding and degradation, they were incubated for 30 min at different temperatures and analyzed by native PAGE (Fig. S8). With human pre-$tRNA^{Tyr}$, the intensity of the main band became weaker at 64 °C and 73 °C (Fig. S8A), suggesting that human pre-$tRNA^{Tyr}$ partly unfolded resulting in its degradation at 64 °C. Analysis of Tth pre-$tRNA^{Gly}$ and Bs pre-$tRNA^{Asp}$ on native PAGE suggested that they remained largely intact at 73 °C (Figs. S8B and S8C), indicating that the two pre-tRNAs are more thermally stable than human pre-$tRNA^{Tyr}$.

We then analyzed the cleavage reaction of pre-tRNA (0.5 μM) with a twofold excess of the Tth ribozyme (1.0 μM). In the prefolding (5 min) followed by the reaction (5 min) at 64 °C, Tth pre-$tRNA^{Gly}$ and Bs pre-$tRNA^{Asp}$ were cleaved efficiently by the Tth ribozyme, whereas human pre-$tRNA^{Tyr}$ showed inefficient cleavage (Fig. S9A). In prefolding and reaction at 70 °C, human pre-$tRNA^{Tyr}$ yielded no product band, whereas the remaining two pre-tRNAs still yielded the product bands (Fig. 4A). These observations indicated that reduction of the cleavage efficiency of human pre-$tRNA^{Tyr}$ at 64 °C and 70 °C was probably due to unfolding of the substrate RNA. The cleavage products of the other two pre-tRNAs disappeared nearly completely in the reaction at 71 °C (Fig. S9B) and disappeared completely at 72 °C (Fig. 4B), suggesting that either the pre-RNAs or the Tth ribozyme RNA started to unfold around 71 °C. We then examined the effects of PEG 8000 and K10 peptide on the reactions. In the absence of PEG 8000, the Tth ribozyme was active at 75 °C (Fig. 4C), indicating that PEG 8000 modestly reduced thermal stability of tertiary structures of the Tth pre-$tRNA^{Gly}$ or Tth RNase P ribozyme (*ca.* 4 °C) at high temperatures over 70 °C. In the absence of PEG 8000, the cleavage reaction was also observed up to 75 °C in the reaction of Bs pre-$tRNA^{Asp}$ (Fig. S9E). To compare thermostability of three RNase P ribozymes, we examined the Ec and Tm ribozymes in the presence and absence of PEG 8000.

In the presence of PEG 8000, the Ec and Tm ribozymes were

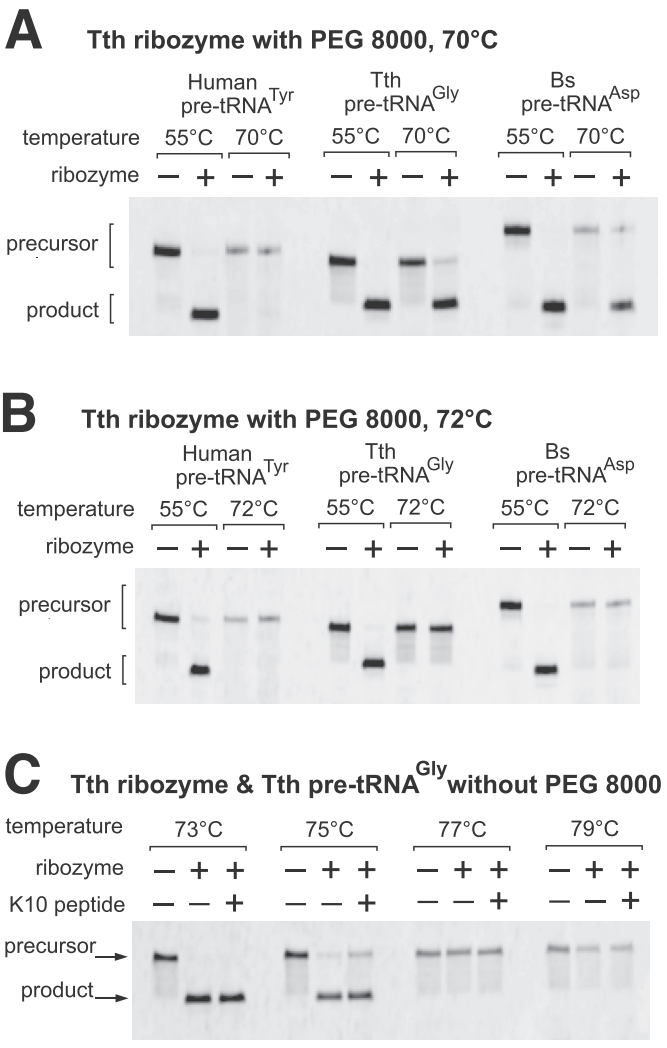

Fig. 4. The upper temperature limits of the RNaseP-catalyzed pre-tRNA cleavage reactions. Reactions were performed with 50 mM Tris-HCl (pH 7.5) and 50 mM $MgCl_2$ in the presence of 0.5 μM pre-tRNA and 1.0 μM Tth RNase P RNA.
A, B) Site-specific cleavage reactions of the three pre-tRNAs catalyzed by the Tth RNase P ribozyme in the presence of PEG 8000.
C) Site-specific cleavage reactions of Tth pre-$tRNA^{Gly}$ catalyzed by the Tth RNase P ribozyme in the absence of PEG 8000.

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active up to 63 °C and 65 °C, respectively (Fig. S9F). In the absence of PEG 8000, they were active up to 67 °C (Figs. S9G and S9H).

4. Discussion

We characterized three bacterial RNase P ribozymes to determine how this class of ribozyme works efficiently even under thermophilic conditions. The most important finding from comparative analysis of these homologous A-type ribozymes was the remarkable activity and thermostability of the Tth ribozyme. The Tth ribozyme showed distinctly higher turnover ability at 55 °C with PEG 8000 and maintained its activity at 75 °C without PEG 8000. Tertiary structures and folding of the Tth and Tm ribozymes seemed to be adapted to thermophilic conditions. On the other hand, the Ec ribozyme was less thermostable but its tertiary structure and folding seemed to be adapted to a broader range of solution temperatures. As the secondary structures of the Tth and Ec ribozymes are highly similar, the molecular mechanism underlying their distinct catalytic and folding properties may be elucidated by construction of chimeric ribozymes [15,25,26]. It will also be interesting to examine rational and evolutionary improvement of the Tth ribozyme to further increase its thermotolerance. In this line of experiments, coevolutionary improvements may also be needed for the thermostability of its substrate pre-tRNAs. These experiments may also shed light on the emergence and evolution of RNA-based enzyme machinery that was functional in the LUCA.

Declaration of competing interest

The authors have no conflicts of interest directly relevant to the content of this article.

Acknowledgements

This work was supported by MEXT KAKENHI Grant Number JP15K05561 (Y.I.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.12.056.

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