Identification by Mn$^{2+}$ rescue of two residues essential for the proton transfer of tRNase Z catalysis

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ABSTRACT

Thermotoga maritima tRNase Z cleaves pre-tRNAs containing the $\gamma_4$CCA$_{75}$ sequence precisely after the $A_{76}$ residue to create the mature $3'$ termini. Its crystal structure has revealed a four-layer $\alpha\beta/\beta\alpha$ sandwich fold that is typically found in the metallo-$\beta$-lactamase superfamily. The well-conserved six histidine and two aspartate residues together with metal ions are assumed to form the tRNase Z catalytic center. Here, we examined tRNase Z variants containing single amino acid substitutions in the catalytic center for pre-tRNA cleavage. Cleavage by each variant in the presence of Mg$^{2+}$ was hardly detected, although it is bound to pre-tRNA. Surprisingly, however, Mn$^{2+}$ ions restored the lost Mg$^{2+}$-dependent activity with two exceptions of the Asp52Ala and His222Ala substitutions, which abolished the activity almost completely. These results provide a piece of evidence that Asp-52 and His-222 directly contribute the proton transfer for the catalysis.

INTRODUCTION

Every single tRNA molecule terminates with the sequence CCA (1). This $3'$ terminal sequence is essential for aminoc酰ylation of the tRNAs (2) and for translation on the ribosome (3). The tRNAs are transcribed as larger precursors, which subsequently undergo various processing steps such as removal of $5'$ and $3'$ extra nucleotides to produce mature tRNAs (4). tRNA $3'$ processing endoribonuclease (tRNase Z) is one of the tRNA-maturing enzymes, which removes a $3'$ trailer from pre-tRNA (5–10). Most tRNase Zs cleave pre-tRNAs immediately downstream of a discriminator nucleotide, onto which the CCA residues are added to generate mature tRNA, while Thermotoga maritima tRNase Z cleaves pre-tRNAs containing the $\gamma_4$CCA$_{76}$ sequence precisely after the $A_{76}$ residue to create the mature $3'$ termini (10).

tRNase Zs can be categorized into two groups: a short form (tRNase Z$^S$) that consists of 300–400 amino acids and a long form (tRNase Z$^L$) that contains 800–900 amino acids (11,12). Bacteria and archaea genomes contain a tRNase Z$^S$ gene only, while eukaryotic genomes encode either only tRNase Z$^L$ or both forms. The C-terminal half region of tRNase Z$^L$ has high similarity to the whole region of tRNase Z$^S$, and these regions contain a well-conserved histidine motif, which has been shown to be essential for the Mg$^{2+}$-dependent enzymatic activity by mutational analyses for T.maritima tRNase Z$^S$ (10) and Drosophila melanogaster tRNase Z$^L$ (13).

Sequence analysis has suggested that tRNase Zs belong to the metallo-$\beta$-lactamase superfamily, and the crystal structures of T.maritima, Bacillus subtilis and Escherichia coli tRNase Zs have revealed a four-layer $\alpha\beta/\beta\alpha$ sandwich fold that is typically found in the metallo-$\beta$-lactamase superfamily (14–16). From the structural and functional studies of the superfamily enzymes, it is assumed that the well-conserved six histidine and two aspartate residues together with metal ions also form the tRNase Z catalytic center (Figure 1A and B). It is known that metal ions such as Zn$^{2+}$, Fe$^{3+}$ and Mn$^{2+}$ are required for activities of this superfamily’s enzymes (17–20).

In the structures of the three bacterial tRNase Zs, a unique long flexible arm extending from the core domain has been uncovered, which is thought to be essential for pre-tRNA binding [(15,16,21,22), R. Ishii, M. Nashimoto and S. Yokoyama, unpublished data]. However, little is known about how the exact cleavage site in pre-tRNA is selected and what makes the T.maritima enzyme cleave pre-tRNA after the $\gamma_4$CCA$_{76}$ sequence not after the discriminator.

Many catalytic mechanisms have been proposed for the metallo-$\beta$-lactamase superfamily (17–20) and
metallo-phosphatases (23), in which nucleophilic attack of the carbonyl group of the \(\beta\)-lactam ring or the phosphoryl group by a hydroxyl generated from a metal-ligated water molecule commonly triggers the catalytic reaction. Although a reaction mechanism for pre-tRNA cleavage by tRNase Z has been proposed on the basis of these precedents (15), no direct evidence was shown.

In this report, we examined \textit{T.maritima} tRNase Z variants containing single amino acid substitutions in the catalytic center for pre-tRNA cleavage. Cleavage by each variant in

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**Figure 1.** Structures of \textit{T.maritima} tRNase Z and pre-tRNAs. (A) The amino acid sequences of \textit{T.maritima} (Tma) and \textit{B.subtilis} (Bsu) tRNase Zs are aligned using ClustalW (25) and represented using ESPript (26). The \textit{T.maritima} tRNase Z secondary structures for \(\alpha\)-helices, \(\beta\)-strands and \(\beta\)-helices are indicated by \(\alpha\), \(\beta\) and \(\eta\), respectively. The red box and the blue rectangle denote the conserved and moderately conserved residues, respectively. The regions of the histidine motif, the flexible arm and the mutated amino acid residues (triangle) are shown. (B) Stereoview for the catalytic center of \textit{T.maritima} tRNase Z (14). The two subunits are colored green and pink, respectively. The mutated residues, except His-134, which is disordered, are represented by ball-and-stick models. One unidentified metal ion is depicted as a yellow sphere. (C) \textit{T.maritima} pre-tRNA\(_{\text{Arg}}\)(\(\gamma_4\)CCA\(_{76}\)) and pre-tRNA\(_{\text{Arg}}\)(\(\gamma_4\)GUG\(_{76}\)). The sequence \(\gamma_4\)CCA\(_{76}\) in the former is substituted with \(\gamma_4\)GUG\(_{76}\) in the latter. Cleavage sites are denoted by arrows.
the presence of Mg$^{2+}$ was hardly detected, although it bound to pre-tRNA. Surprisingly, however, Mn$^{2+}$ ions rescued the lost Mg$^{2+}$-dependent tRNase Z activity with two exceptions of the Asp52Ala and His222Ala substitutions. This observation suggests that Asp-52 and His-222 may directly contribute the proton transfer for the catalysis.

**MATERIALS AND METHODS**

**Preparation of the tRNase Z variants**

Based on pQE-80L (Qiagen), the expression plasmids for 10 *T.maritima* tRNase Z variants containing a single amino acid substitution, Phe11Pro, His134Ala, Asp190Ala, His222Ala, His244Ala, Asp52Ser, Asp52Glu, His222Cys, His222Ser or His222Lys, were constructed using specific primer pairs (Supplementary Table 1) as described before (10). Histidine-tagged *T.maritima* tRNase Z variants were produced in *E.coli* cells and purified using nickel–agarose beads as described previously (10).

**Pre-tRNA synthesis**

The *T.maritima* pre-tRNAs were synthesized *in vitro* with T7 RNA polymerase (Takara Shuzo) from the synthetic pre-tDNAs containing its promoter. The transcription reactions were carried out under the conditions recommended by the manufacturer (Takara Shuzo), and the transcribed pre-tRNAs were gel-purified. The pre-tRNAs were subsequently labeled with fluorescein according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Briefly, after the removal of the 5′-phosphates of the transcripts with bacterial alkaline phosphatase (Takara Shuzo), the transcripts were phosphorylated with ATP$\gamma$S and T4 polynucleotide kinase (Takara Shuzo). Then a single fluorescein moiety was appended onto the 5′-phosphorothioate site. The resulting pre-tRNAs with fluorescein were gel-purified before assays.

**In vitro tRNA 3′ processing assay**

The 3′ processing reactions for fluorescein-labeled pre-tRNA were performed with the *T.maritima* tRNase Z variants basically in a mixture (6 μl) containing 10 mM Tris–HCl (pH 7.5), 1.5 mM DTT, 25 mM NaCl and 10 mM MgCl$_2$ (or 0.2 mM MnCl$_2$) at 60°C. In several assays, pH and temperature were changed or different divalent metal ions were used. After resolution of the reaction products on a 10% denaturing polyacrylamide gel with TBE buffer (90 mM Tris–HCl (pH 8.3), 90 mM boric acid and 1.5 mM EDTA, pH 8.3), alkaline phosphatase (Takara Shuzo), the transcripts were phosphorylated with ATP$\gamma$S and T4 polynucleotide kinase (Takara Shuzo), the transcripts were phosphorylated with ATP$\gamma$S and T4 polynucleotide kinase (Takara Shuzo). Then a single fluorescein moiety was appended onto the 5′-phosphorothioate site. The resulting pre-tRNAs with fluorescein were gel-purified before assays.

**RESULTS AND DISCUSSION**

**Metal ion requirements for the *T.maritima* tRNase Z activity**

First of all, we examined metal ion requirements for the *T.maritima* tRNase Z activity. *T.maritima* pre-tRNA$^{\text{Avr} (74\text{CCA}_{76})}$ 5′ end labeled with fluorescein (Figure 1C) was reacted with the wild-type enzyme in the presence of several metal ions. Mg$^{2+}$ and Mn$^{2+}$ ions activated the enzyme in the range of 0.2–10 mM, while Zn$^{2+}$ did not at all activate in the range of 0.02–10 mM (Figure 2A). Although this enzyme was active in the presence of 0.2 or 10 mM Mg$^{2+}$ alone (Figure 2A), the activity decreased with the increase in the added Zn$^{2+}$ amount and vanished in the presence of high amounts of Zn$^{2+}$ (Figure 2B). Similar result was obtained with respect to the Mn$^{2+}$-dependent activity (Figure 2B). These data indicate that Mg$^{2+}$ or Mn$^{2+}$ ions are essential for the *T.maritima* tRNase Z activity and that Zn$^{2+}$ is inhibitory to the activity in high concentrations. A similar observation with respect to *Arabidopsis thaliana* tRNase Z has been reported (24).

We have identified two zinc ions in the active site of the crystal structure of *T.maritima* tRNase Z ([R. Ishii, M. Nashimoto and S. Yokoyama, unpublished data]. Similarly, two zinc ions in the active site have been detected in tRNase Zs from *B.subtilis* and *E.coli* (15,16). One zinc ion was also coordinated in the *B.subtilis* enzyme/tRNA complex recently uncovered (21). Although these observations appear to be discrepant with the above results, the coordinated Zn$^{2+}$ ions may be needed for the activity. Alternatively, Zn$^{2+}$ ions may be dispensable for the active form of tRNase Z. In any case, the roles of Mg$^{2+}$ or Mn$^{2+}$ ions would be to help fold pre-tRNA properly, to induce tRNase Z to form the active conformation, and/or to assist tRNase Z to interact with pre-tRNA correctly.

**Mn$^{2+}$ rescue of the tRNase Z activity**

Next, we examined how single amino acid substitutions with Ala in the catalytic center other than the histidine motif affect pre-tRNA cleavage by *T.maritima* tRNase Z (Figure 1A and B). Recombinant histidine-tagged tRNase Z variants containing a single substitution, His134Ala, Asp190Ala, His222Ala or His244Ala (Figure 1A and B) were also examined (10). As shown in Figure 3A, all these variants hardly cleaved pre-tRNA$^{\text{Avr} (74\text{CCA}_{76})}$ in the presence of Mg$^{2+}$, although they bound to the substrate with varying $K_d$ values (0.02–1.40 μM) (Table 1). The cleavage of pre-tRNA$^{\text{Avr} (74\text{GUG}_{76})}$ in the presence of Mg$^{2+}$ was also hardly detected, and the enzymes bound to the substrate with varying $K_d$ values (0.02–1.66 μM) (Table 1). These results confirm that the six histidines (amino acids 48, 50, 53, 134, 222 and 244) and two aspartates (amino acids 52
and 190) are important for catalysis, and suggest that these residues are not essential for pre-tRNA binding although some of these may participate in some of the important interactions.

These variants were also tested for cleavage of these pre-tRNAs in the presence of Mn\textsuperscript{2+}. To our surprise, Mn\textsuperscript{2+} ions restored the Mg\textsuperscript{2+}-dependent tRNase Z activity lost by single His or Asp substitutions with two exceptions (Asp52Ala and His222Ala), although the observed rate constant values differed depending on the variants and the substrates (Figure 3B and Table 1). We confirmed that the Mn\textsuperscript{2+}-rescued activity of these variants is endoribonucleolytic not exoribonucleolytic (data not shown).

Zn\textsuperscript{2+}, Cu\textsuperscript{2+} and Co\textsuperscript{2+} ions cannot rescue the lost tRNase Z activity

We also examined other metal ions for the ability to rescue the lost Mg\textsuperscript{2+}-dependent activity of the tRNase Z variants. Two representative tRNase Z variants, His50Ala and Asp52Ala, were assayed in the presence of Zn\textsuperscript{2+}, Cu\textsuperscript{2+} or Co\textsuperscript{2+}. None of these metal ions rescued the lost tRNase Z activity in the ion concentration of 0.2 mM (data not shown). These metal ions did not rescue also in the range of 0.1–10 mM (data not shown).

Metal ion coordination in tRNase Z

Together with the observation that Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions are indispensable for the T.maritima tRNase Z activity, the Mn\textsuperscript{2+}-rescue phenomena imply that, instead of Zn\textsuperscript{2+} ions, Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions may be coordinated in the catalytic center of T.maritima tRNase Z. This coordination may be limited in the active enzyme/substrate complex. Without the substrate, Zn\textsuperscript{2+} ions may be weakly coordinated in the catalytic center, and, upon pre-tRNA binding, Zn\textsuperscript{2+} ions may be replaced with Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions. This supposition appears to be consistent with the observation that only one Zn\textsuperscript{2+} ion is coordinated in the active site of the crystal structure of the B.subtilis tRNase Z/tRNA complex, whereas two Zn\textsuperscript{2+} ions are coordinated in the active site of the free protein structure (15,21). Alternatively, Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions may be coordinated near the catalytic center to induce an active enzyme/substrate conformation. These possibilities are not mutually exclusive. High resolution crystal structure of the pre-tRNA/tRNase Z complex in the presence of the Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions would clarify this issue.
The Mn\(^{2+}\) rescue phenomena would be based primarily on the property that Mn\(^{2+}\) ions have higher affinity to the active site and/or a nearby site than Mg\(^{2+}\). Mn\(^{2+}\) ions would be able to be coordinated even in the catalytic site and/or the nearby site distorted due to the lack of one of the important residual groups.

### Existence of specific interactions with the CCA bases

The Mn\(^{2+}\)-rescued cleavage of pre-tRNA\(^{AGG}\)(\(7_{4}CCA_{76}\)) by the variants occurred at the same site as by the wild type, while the cleavage site of pre-tRNA\(^{AGG}\)(\(7_{4}CCA_{76}\)) by each enzyme fluctuated by one nucleotide (Table 1). The cleavage by the variants His48Ala, His50Ala, Asp190Ala, and His244Ala occurred primarily after CCA, and the cleavage by the other variants was primarily after CC as in the case of the wild type (Table 1). The cleavage of pre-tRNA\(^{AGG}\)(\(7_{4}GUG_{76}\)) by the former variants was ~7-fold less efficient than that of pre-tRNA\(^{AGG}\)(\(7_{4}CCA_{76}\)), suggesting that these residues are important for the correct interaction with the nucleotide residues near the scissile bond of CCA-less pre-tRNA and that there would exist other residues that interact specifically with the bases of the CCA nucleotides.

### His-134 may be important to grasp pre-tRNA

The observation that the cleavage site changes by one nucleotide depending on the mutation site, the nucleotide residues near the scissile bond, and the metal ion species suggests that, upon binding of pre-tRNA, the enzyme would change its conformation into the activated form accommodating the two metal ions. Furthermore, together with the fact that His-134 is disordered in the crystal (14), the observation that the His134Ala variant has a 28-fold higher \(K_d\) value than the wild type in the interaction with pre-tRNA\(^{AGG}\)(\(7_{4}CCA_{76}\)) (Table 1) implies that, in the Mn\(^{2+}\)-dependent reaction, His-134 may play an important role in clamping pre-tRNA.

### Mn\(^{2+}\)-rescue of Phe11Pro and Asp25Ala variants

We examined how a single substitution of Phe-11 or Asp-25, which resides in the peripheral region of the active site, affects the tRNAse Z activity. Phe-11 was substituted with Pro that is highly conserved among the other tRNAse Zs and Asp-25, which is also highly conserved and appears to fix the active site pocket, was replaced with Ala. These variants were not able to cleave both pre-tRNA\(^{AGG}\) substrates in the presence of Mg\(^{2+}\) although they bound to the substrates with \(K_d\) values of 0.27–0.41 \(\mu\)M (Figure 3A and Table 1). Interestingly, the Mg\(^{2+}\)-dependent activity lost by either single substitution was also restored by Mn\(^{2+}\) ions (Figure 3B and Table 1). The other metal ions Zn\(^{2+}\), Cu\(^{2+}\) and Co\(^{2+}\) did not rescue the lost tRNAse Z activity of the Asp25Ala variant in the ion concentrations of 0.1–10 \(\mu\)M (data not shown). Their \(k_{\text{obs}}\) values (0.12–0.26 min\(^{-1}\)) were comparable to those (0.20 and 0.24 min\(^{-1}\)) of the wild type and their cleavage sites were the same as those of the wild type (Table 1). This observation indicates that the Mn\(^{2+}\)-rescue phenomena are not restricted to the variants containing active site residue substitutions and occurs also in the variants with substitutions in the peripheral region of the active site. Mn\(^{2+}\) ions but not Mg\(^{2+}\) ions would be able to be coordinated even in the catalytic site and/or the nearby site distorted due to the substitution of one of the peripheral residues that affect the active site structure.

### Table 1. Dissociation constants and observed rate constants for *T. maritima* tRNAse Z variants

| Enzyme   | Ion       | Pre-tRNA\(^{AGG}\)(\(7_{4}CCA_{76}\)) Cleavage | \(K_d\) (\(\mu\)M)\(^a\) | \(k_{\text{obs}}\) (min\(^{-1}\))\(^b\) | Pre-tRNA\(^{AGG}\)(\(7_{4}GUG_{76}\)) Cleavage | \(K_d\) (\(\mu\)M)\(^a\) | \(k_{\text{obs}}\) (min\(^{-1}\))\(^b\) |
|----------|-----------|-----------------------------------------------|-----------------|------------------|-----------------------------------------------|-----------------|------------------|
| Wild type | Mg\(^{2+}\) | CCAl                                         | 0.05 ± 0.012    | 0.15 ± 0.010     | GUIG                                         | 0.17 ± 0.035    | 0.08 ± 0.001     |
|          | Mg\(^{2+}\) | CClA                                         | 0.09 ± 0.010    | 0.24 ± 0.031     | GUIG                                         | 0.16 ± 0.009    | 0.20 ± 0.028     |
| F11P     | Mg\(^{2+}\) | —                                            | 0.27 ± 0.069    | —                | GUIG                                         | 0.41 ± 0.032    | —                |
| D25A     | Mg\(^{2+}\) | —                                            | 0.15 ± 0.036    | 0.13 ± 0.004     | GUIG                                         | 0.12 ± 0.025    | 0.12 ± 0.006     |
| D52A     | Mg\(^{2+}\) | —                                            | 0.30 ± 0.074    | —                | GUIG                                         | 0.29 ± 0.081    | —                |
| H48A     | Mg\(^{2+}\) | —                                            | 0.03 ± 0.004    | —                | GUIG                                         | 0.11 ± 0.021    | —                |
| H50A     | Mg\(^{2+}\) | —                                            | 0.29 ± 0.082    | —                | GUIG                                         | 0.35 ± 0.006    | —                |
| D190A    | Mg\(^{2+}\) | —                                            | 0.44 ± 0.082    | —                | GUIG                                         | 1.34 ± 0.392    | —                |
| H222A    | Mg\(^{2+}\) | —                                            | 0.16 ± 0.095    | —                | GUIG                                         | 0.19 ± 0.020    | —                |
| H244A    | Mg\(^{2+}\) | —                                            | 0.06 ± 0.010    | —                | GUIG                                         | 0.12 ± 0.018    | —                |
| H134A    | Mg\(^{2+}\) | CCAl                                         | 1.40 ± 0.069    | —                | GUIG                                         | 1.66 ± 0.286    | —                |
| D190A    | Mg\(^{2+}\) | CCAl                                         | 0.22 ± 0.024    | —                | GUIG                                         | 0.13 ± 0.031    | —                |
| H134A    | Mg\(^{2+}\) | CCAl                                         | 0.42 ± 0.015    | —                | GUIG                                         | 0.28 ± 0.019    | —                |
| H222A    | Mg\(^{2+}\) | CCAl                                         | 0.08 ± 0.021    | —                | GUIG                                         | 0.20 ± 0.026    | —                |
| H244A    | Mg\(^{2+}\) | CCAl                                         | 0.05 ± 0.025    | —                | GUIG                                         | 0.07 ± 0.013    | —                |
| H244A    | Mg\(^{2+}\) | CCAl                                         | 0.17 ± 0.015    | —                | GUIG                                         | 0.33 ± 0.138    | —                |

\(^a\)Data are the means ± SD of three independent experiments.

\(^b\)Each fluorescein-labeled pre-tRNA (20 nM) was reacted with each variant (700 nM) at 60°C for 3 min. Data are the means ± SD of three independent experiments.

**Note:** Na\(^{+}\) and K\(^{+}\) ions have higher affinity to the active site. Thus, the Mn\(^{2+}\)-rescue phenomena would be based primarily on the property that Mn\(^{2+}\) ions have higher affinity to the active site and/or a nearby site than Mg\(^{2+}\). Mn\(^{2+}\) ions would be able to be coordinated even in the catalytic site and/or the nearby site distorted due to the lack of one of the important residual groups.
Asp-52 and His-222 may directly contribute the proton transfer

Curiously, the Asp-52 or His-222 substitution with Ala abolished the enzymatic activity almost completely even in the presence of Mn$^{2+}$ (Figure 3A and B and Table 1). This abolished activity was not due to the loss of the pre-tRNA binding ability because the His222Ala variant bound to both pre-tRNAs very stably and the Asp52Ala did so fairly well (Table 1). These results imply that Asp-52 and His-222 not only support the active center structure but also may directly contribute the proton transfer for the catalysis.

The indispensability of Asp-52 is consistent with a catalytic mechanism proposed for B.subtilis tRNase Z, in which Asp-67 (Asp-52 in the T.maritima enzyme) acts as a general base to create a hydroxide ion that attacks the phosphate group (15). In this model, a proton is donated from a water molecule to stabilize the 3'$^{\circ}$ oxygen generated by the phosphodiester bond cleavage. However, the absence of the Mn$^{2+}$ rescue for the His222Ala variant together with the observations that His-247 (His-222 in the T.maritima enzyme) of B.subtilis tRNase Z is located near the scissile bond and that His-222 of the T.maritima enzyme is positioned similarly suggests that this histidine may act as a proton donor. Similarly, it is proposed that the equivalent His in the phosphorylcholine esterase domain of choline-binding protein E plays a catalytic role (20).

Furthermore, if this supposition is correct, the enzymes containing substitutions of Asp-52 with Glu ($pK_a$ 4.2) and of His-222 with Cys ($pK_a$ 8.3) or Lys ($pK_a$ 10.0) should be active in the presence of Mn$^{2+}$ even though they would have no activity in the presence of Mg$^{2+}$ ions. These variants, which were expressed in E.coli and purified with nickel agarose (Figure 4A), were tested for cleavage of pre-tRNA$^{Arg}$ (74CCA76), and this was indeed the case (Figure 4B and Table 2). As expected, Mn$^{2+}$-rescued activity was hardly detected with respect to substitutions of Asp-52 with Ser and His-222 with Ser, although these variants bound to the substrate fairly well (Figure 4B and Table 2).

The $pK_a$ value of Glu (4.2) is almost the same as that of Asp (3.9), whereas the $pK_a$ values of Cys (8.3) and Lys (10.0) are much higher than that of His (6.0). Thus, pH-spectra of the $pK_a$ values of Cys (8.3) and Lys (10.0) are much higher than that of His (6.0). Thus, pH-spectra of the tRNase Z activity for the His222Cys and His222Lys variants would be changed and the optimal pH would be shifted to a higher value, if His-222 is a proton donor. We measured the pH-spectra for the wild type and the His222Lys variant. As expected, the pH-spectrum for the variant drastically changed and the optimal pH value shifted from 8.0 for the wild type to 9.0 for the variant (Figure 5). The activity of the His222Cys variant was too low to accurately obtain a pH-spectrum. Taken together, these data strongly suggest that Asp-52 and His-222 may act as a general base and a proton donor, respectively.

A possible catalytic mechanism of tRNase Z

Based on the present results, we propose a mechanism for the tRNase Z catalysis (Figure 6). The chemistry of the

![Figure 4](https://academic.oup.com/nar/article-abstract/34/13/3811/1157969)

**Figure 4.** The Mn$^{2+}$-rescue assays for T.maritima tRNase Z variants containing single substitutions at Asp-52 or His-222. (A) Protein profiles of histidine-tagged T.maritima tRNase Z variants. Each purified protein (0.5–1 $\mu$g) was analyzed on an SDS–10% polyacrylamide gel, and visualized by staining the gel with Coomassie brilliant blue R-250. (B) The assays for in vitro 3'$^\prime$ processing of T.maritima pre-tRNA$^{Arg}$ (74CCA76) by T.maritima tRNase Z variants. The pre-tRNA (0.1 pmol) was incubated with the tRNase Z variants (4 pmol) at 60°C for 10 min. The cleavage products were analyzed on a 10% polyacrylamide–8 M urea gel.
phosphodiester bond cleavage reaction would begin with deprotonation and activation of a metal-coordinated water molecule by the Asp-52 residue to create a nucleophile. The resulting hydroxide ion would attack the phosphate group that connects tRNA and 3′ trailer sequences, and would cleave the phosphodiester bond leaving the phosphate at the 5′ terminus of the trailer. The reaction would finish with protonation of the 3′ oxygen of the tRNA molecule by the His-222 residue. Mg2+ or Mn2+ ions would help position the scissile phosphate properly in the active site through bridging oxygens of the pre-tRNA backbone phosphates and carbonyl oxygens of tRNase Z.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

| Enzyme | Ion   | Pre-tRNA<sub>Arg</sub><sub>(74CCA76)</sub> Cleavage | K<sub>d</sub> (μM)<sup>a</sup> | k<sub>obs</sub> (min<sup>-1</sup>)<sup>b</sup> |
|--------|-------|---------------------------------|----------------|-----------------|
| D52S   | Mg<sup>2+</sup> | —                              | 0.35 ± 0.083 | —               |
|        | Mn<sup>2+</sup> | —                              | 0.29 ± 0.018 | —               |
| D52E   | Mg<sup>2+</sup> | —                              | 0.29 ± 0.160 | —               |
|        | Mn<sup>2+</sup> | CClA                            | ND             | 0.36 ± 0.004   |
| H222C  | Mg<sup>2+</sup> | —                              | 0.39 ± 0.095  | —               |
|        | Mn<sup>2+</sup> | CClA                            | ND             | <0.01           |
| H222S  | Mg<sup>2+</sup> | —                              | 0.36 ± 0.094  | —               |
|        | Mn<sup>2+</sup> | —                              | 0.25 ± 0.087  | —               |
| H222K  | Mg<sup>2+</sup> | —                              | 0.23 ± 0.020  | —               |
|        | Mn<sup>2+</sup> | CClA                            | ND             | 0.06 ± 0.003   |

<sup>a</sup> Data are the means ± SD of three independent experiments (Supplementary Figure 1).
<sup>b</sup> Each fluorescein-labeled pre-tRNA (20 nM) was reacted with each variant (700 nM) at 60°C for 3 min. Data are the means ± SD of three independent experiments.

Figure 5. pH-spectra of the tRNase Z activity. Relative k<sub>obs</sub> values for the T.maritima pre-tRNA<sub>Arg</sub><sup>74CCA76</sup> cleavage reaction by T.maritima wild-type tRNase Z and its H222K variant are plotted against pH. The fluorescein-labeled pre-tRNA (20 nM) was reacted with each variant (700 nM) at pH 7–10 at 25°C.

Figure 6. A possible mechanistic scheme for pre-tRNA cleavage by T.maritima tRNase Z. Asp-52 works as a proton acceptor and His-222 acts as a proton donor. R1, upstream tRNA residue; R2, downstream trailer residue. See text for a detailed description.

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