Analysis of the Functional Modules of the tRNA 3′ Endonuclease (tRNase Z)*

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Precursors, which do contain the CCA, are not processed by tRNase Z. Archaea and eukaryotes also use tRNase Z enzymes to process the tRNA 3′ trailer in a single-step mechanism (5–8).

The first tRNase Z, TRZ1, was isolated from Arabidopsis thaliana (5). Data base analyses showed that TRZ1 homologues are present in organisms from all three kingdoms, bacteria, archaea, and eukarya (Fig. 1). The tRNase Z family of proteins (also called Elac1/Elac2) can be divided into two subgroups: the short tRNase Z proteins (being 250–350 amino acids long), tRNase Z§ enzymes, and the long tRNase Z proteins (with 700–950 amino acids), the tRNase Z^ enzymes. Although the tRNase Z§ enzymes are present in all kingdoms, the tRNase Z^ enzymes can only be found in eukarya. Both subgroups are part of the same protein family since the C-terminal part of the tRNase Z^ proteins has high sequence similarity to the tRNase Z§ enzymes.

TRZ1 belongs to the family of metal-dependent β-lactamases (9), a group of metalloproteins that perform a variety of diverse functions (10–12). This metalloprotein family was classified into 16 subgroups (12), and the tRNase Z enzymes are part of the Elac1/Elac2 subgroup. Other subgroups include the 3′ mRNA cleavage and adenylation specificity factors (13), SNM1 (also named PSO2), and Artemis (14, 15), proteins that are involved in DNA repair (16). Another subgroup consists of cAMP phosphodiesterase enzymes (17), which catalyze the hydrolysis of cAMP to the corresponding nucleoside 5′-monophosphate. The class II cAMP phosphodiesterase enzymes have been shown to bind two Zn^2+ ions (17). In general, metallo-β-lactamases bind one or two metal ions, preferably zinc, iron, or manganese (10). The E. coli tRNase Z enzyme has been shown to bind two zinc ions (18, 19).

The recently published crystal structure of the tRNase Z enzymes from B. subtilis (20) and Thermotoga maritima (21) confirms that the tRNase Z enzymes belong to the family of metal-dependent β-lactamases since they contain the metallo-β-lactamase fold. Structural data also show that the enzyme is a homodimer with the monomers arranged head to head. The two monomers jointly form the active site cleft, which can readily accommodate single-stranded RNA. The exosite (an element outside the active site that participates in substrate binding) protrudes from the main protein body pointing toward the solvent.

We are currently analyzing the functional modules of the eukaryotic tRNase Z enzyme, TRZ1, from A. thaliana. It is a short tRNase Z enzyme of 280 amino acids containing two potential leucine zippers and a histidine motif that is part of a metallo-β-lactamase motif containing 3 highly conserved histidine residues. To identify amino acids involved in dimerization, tRNA binding, and catalysis, 24 point mutations were made. In addition, several deletion variants were generated that have one of the potential motifs removed or carry a deletion from the C terminus.

Our results show that several conserved amino acids can be mutated without or with little loss of tRNA processing activity. We identify 4 conserved amino acids to be required for dimerization and 1 amino acid...
and three regions important for tRNA binding. In addition, 6 amino acids were shown to be required for catalytic activity.

EXPERIMENTAL PROCEDURES

Mutagenesis Strategy—The TRZ1 cDNA was excised from pET32a-nuz (5) (using NcoI and XhoI) and cloned into pBlue KSII (digested with NcoI and XhoI) yielding pBlue-nuz. Inverse PCR was employed using pBlue-nuz as template to generate the TRZ1 variants. For deletion variants, the primers (primer sequences are available upon request) spared the region to be deleted. For point variants, one of the primers carried the mutation. PCR products were ligated to yield the pBlue-mutant clones. Depending on the nature of the amino acids to be mutated, the mutation. PCR products were ligated to yield the pBlue-mutant clones. Depending on the nature of the amino acids to be mutated, the resulting amino acids were one of the hydrophobic amino acids, glycine, alanine, leucine, valine, isoleucine, and serine. The pBlue-mutant clones were digested with NcoI and XhoI to release the cDNA and the mutated cDNA was subcloned into pET32a and pET29a (Novagen), respectively.

Cross-linking Assay—For cross-linking assays with glutaraldehyde, 1 µg of protein was incubated with glutaraldehyde (final concentration 0.05%) in double distilled water in 10 µl for 30 min at room temperature. After the addition of 1 µl of 1 M lysine, the reaction was incubated for 20 min at room temperature. Finally, 2 µl of 1 M lysine, the sample was loaded onto a SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Hybond C, Amersham Biosciences). TRZ1 monomers and homodimers were detected using a primary rabbit antibody against TRZ1.

Electrophoretic Mobility Shift Assay—tRNA from wheat (tRNA isolated from wheat, Type V, Sigma) was 3’-end-labeled with [32P]Pcpp. 1 fmol of labeled tRNA was heated for 5 min at 80 °C in binding buffer (10 mM Tris, pH 8.0, 5 mM MgCl2, 10 mM KCl, 10 µg/ml bovine serum albumin, and 5% glycerol) and allowed to cool down to room temperature. Recombinant TRZ1 (100 ng), phenylmethylsulfonyl fluoride, and dithiothreitol were added (to a final concentration of 0.5 and 1 mM, respectively), and the reaction was incubated for 20 min at room temperature. Finally, 2 µl of 100% glycerol was added, and the reaction was loaded onto 8% native PAGE, which was run 60 min at 4 °C with 10 V/cm in TG buffer (10 mM Tris, pH 8, 58 mM glycine). Gels were analyzed by autoradiography.

Substrate Preparation—Precursor tRNA<sup>TPY</sup> from Oenothera bicerrifera was transcribed from template pTyrII as described (23).

In Vitro Processing Assays—All processing assays were carried out with 100 ng of recombinant protein in a reaction volume of 100 µl in nuz-IVP buffer (40 mM Tris, pH 8.4, 2 mM MgCl2, 2 mM KCl, and 2 mM dithiothreitol) at 37 °C for 30 min. To investigate the effect of imidazole upon the reaction, imidazole (1 and 10 mM final concentration) was added to the reaction mixture. Processing reactions were terminated by phenol and chloroform extractions. Nucleic acids were precipitated, and reaction products were analyzed on 8% polyacrylamide gels. Gels were analyzed by autoradiography.

Metal Dependence of the in Vitro Processing Reaction—To analyze which metal ions are required for the processing reaction, reactions were preincubated for 5 min at 37 °C with 1 and 10 mM 1,10-phenanthroline or for 1 h at 4 °C with 10 mM EDTA. Chelators and chelator-metal complexes were subsequently removed by dialysis.
RESULTS

Gel filtration analyses suggested that TRZ1 might be active as a homodimer. Thus we performed cross-linking assays with the recombinant TRZ1 and all variants to identify potential multimers. To investigate how tightly TRZ1 binds to the substrate and/or to the product, we performed electrophoretic mobility shift assays with TRZ1 and all variants.

As shown previously, the recombinant tRNase Z TRZ1 from A. thaliana cleaves tRNA precursors efficiently in vitro (5). Thus as a test for catalytic activity, all variants were incubated with precursor tRNAs.

Characteristics of the Wild Type Protein TRZ1—Cross-linking experiments with TRZ1 showed that the enzyme is indeed a homodimer (Fig. 2). Electrophoretic mobility shift assay analyses revealed that TRZ1 binds tightly to tRNA molecules but only weakly to the synthetic precursor tRNA (Fig. 3 and data not shown). The fact that TRZ1 did not bind well to the precursor tRNA under the conditions employed could be due to the fact that the precursor RNA used was made in vitro, and thus tRNA nucleotides were not modified and the precursor molecules might not fold correctly. Thus the following RNA binding studies were made with wheat tRNA. Incubation with precursor tRNA formed the catalytic activity of TRZ1 (Fig. 4).

Rationale for Mutant Selection—To identify important regions and amino acids that we chose for mutations (Fig. 1, TABLE ONE). Motif search software predicted three regions to be a histidine motif and two potential leucine zippers, respectively. Therefore we made three internal deletions: one, del51–60, which spans the histidine motif, and the other two, del149–164 and del200–212, which span the two potential leucine zippers (starting and ending with the 1st and 3rd leucine). In addition, we made a deletion from the C terminus (del270–280) to define a shorter tRNase Z enzyme.

Point variants were generated of amino acids conserved between tRNase Z proteins (Fig. 1). Of particular interest were the amino acids from the histidine motif, which are conserved between the metal-dependent β-lactamases (His-54, His-56, Asp-58, and His-59) and the two histidines (His-133 and His-226) that might be involved in metal binding (11). In addition to mutation of conserved amino acids, we also mutated 3 amino acids that are specific for TRZ1. Cys-25 and Cys-40 can only be found in the TRZ1 sequence and could be involved in dimer formation. Lys-203 was also unique for the TRZ1 sequence and was at a position where all other tRNase Z proteins have a conserved aspartic acid. All TRZ1 variants were expressed at the same levels as wild type TRZ1 in soluble form and purified with S protein-agarose. The E. coli
protein GroEL (22) copurified with all recombinant proteins, but the wild type protein is not influenced by the presence of GroEL in any of the three activities tested (dimerization, tRNA binding, and processing). Thus only class 3 variants were separated from GroEL using anion exchange chromatography yielding pure proteins to confirm that the loss of processing activity in these variants was not due to the presence of GroEL. Indeed, activity tests with variants with and without GroEL gave the same results. Only in a few cases (Pro-83, Thr-186, del270–280) was it not possible to separate GroEL from the TRZ variants. Interestsionally, these proteins were also not able to form dimers. GroEL does not interfere with dimerization since the other proteins dimerize also in the presence of GroEL. All variants were analyzed in respect to their ability to form dimers, to bind to tRNA, and to catalyze tRNA processing.

During this study, structures of two bacterial tRNase Z enzymes have been solved. To discuss the results of this mutational study in a structural context, all mutations were mapped onto the B. subtilis structure based on the tRNase Z alignment (Fig. 5).

**Metal Ions Are Required for tRNA Processing Activity**—It was shown previously that metal ions are required for tRNA processing activity of TRZ1 since preincubation with EDTA resulted in loss of activity (24). Interestingly, the metal required seems to be already bound by the enzyme since the addition of metal ions to the recombinant enzyme after expression in E. coli is not necessary. To elucidate which metal is required for activity, we preincubated the reaction with chelators 1,10-phenantroline and EDTA (Fig. 6). Preincubation with 10 mM 1,10-phenantroline and EDTA inhibited the reaction. Of several metal ions required for activity, we preincubated the reaction with chelators 1,10-phenantroline and EDTA (Fig. 6). Preincubation with 10 mM 1,10-phenantroline and EDTA inhibited the reaction. Of several metal ions tested, only the addition of Mn²⁺ and Mg²⁺ rescued the activity, showing that these metal ions are required for the activity.

**Removal of Several Internal Amino Acids Leads to Inactivation of the Protein**—Deletions of the histidine motif (del51–60) and the potential leucine zipper motifs (del149–164 and del200–212) (TABLE ONE and Supplemental Figs. 2–4) resulted in almost complete inactivation of the enzyme Fig. 2), that they were not able to bind tRNA anymore, and that they could not process precursor tRNAs.

**The C Terminus Is Essential for tRNA Binding and Processing Activity**—Removal of 11 amino acids at the C terminus resulted in variant del270–280, which does not form dimers but does form multimers. In addition, this variant...
Amino Acids That Are Not Essential for Activity (Class One)—Variants C40G (Figs. 2–4), F51L, P64A, and T210I (Supplemental Figs. 2–4) showed the same activity or nearly the same activity (80–100%) as the wild type protein, suggesting that amino acids Cys-40, Phe-51, Pro-64, and Thr-210 are not essential for dimerization, tRNA binding, and catalysis. Amino acid Cys-40 was not conserved throughout the tRNase Z proteins and present only in TRZ1 from Arabidopsis; therefore it was not too surprising that mutation of that amino acid does not interfere with activity. However, Phe-51, Pro-64, and Thr-210 are conserved in at least four of the five protein sequences aligned in Fig. 1; thus it is interesting that these amino acids do not seem to be essential for processing activity.

Variants That Do Not Have Catalytic Activity (Class Three)—Variants H54L (Figs. 2–4), H56L, D58A, H133L, D185G, H226L, and delR252 (Supplemental Figs. 2–4) still form dimers and bind to tRNA but are not able to process precursor tRNAs. However, they cleave tRNA precursors with reduced activity (20–80% wild type activity, TABLE ONE). Since these variants all still formed dimers and bind to tRNAs, the mutation seemed to affect only the catalytic activity.

In the case of ribozyme-mutants, which were not catalytically active anymore, the addition of imidazole rescued the catalytic activity (25). To test whether the addition of imidazole to the histidine mutants can rescue the catalytic activity, we added 1 and 10 mM imidazole to the processing reaction with mutants H54L, H56L, Asp-58, His-133, Asp-185, and His-226 in catalysis. The mutation of amino acid R252G only reduced the catalytic activity (down to 26%, see above); deletion of this amino acid, however, resulted in total loss of activity.

DISCUSSION

Although tRNA 3′ end processing is vital for tRNA maturation and subsequent aminoacylation, little is known about the functional domains of the tRNA 3′-processing enzyme tRNase Z. We performed an extensive mutational study of the tRNase Z from A. thaliana
to identify amino acids and regions important for dimerization, tRNA binding, and catalysis.

During this study, the structures of the bacterial tRNase Z enzymes from *B. subtilis* and *T. maritima* were solved (20, 21). Our results obtained from the mutational study of the eukaryotic short tRNase Z enzyme presented here were thus compared with the bacterial structure.

**Characteristics of the tRNase Z Enzyme From A. thaliana**—The TRZ1 protein is a homodimer, as are the homologous proteins from *E. coli* (18), *T. maritima* (21), and *B. subtilis* (20), and it tightly binds to tRNAs and is catalyzing tRNA 3′ end processing. The crystal structure of the bacterial enzyme shows that the two subunits adopt different conformations in the dimer (20, 21). Thus mutations in the monomeric subunit can have different effects, depending on the function the respective monomer has.

We were not able to construct a smaller TRZ1 version since deletion of 11 amino acids at the C terminus results in loss of activity. It is quite surprising that the deletion at the C terminus inactivates the protein since it is a region where only a few amino acids are conserved between two or three of the aligned protein sequences (Fig. 1). Comparison with the crystal structure of the tRNase Z from *B. subtilis* (20) shows that the C-terminal deletion del270–280 completely removes the β sheet β16 (Fig. 5). Maybe this particular β16 is important to stabilize the structure in that region since it is in proximity to the proposed catalytic center in subunit A (20).

**Changes That Do Not Interfere with Processing**—It is quite surprising that three conserved amino acids (Phe-51, Pro-64, and Thr-210) can be mutated without much reduction of the processing activity (variants have still 80–100% activity when compared with the wild type activity). The change of additional 7 conserved amino acids resulted in only reduced catalytic activity (Gly-62, Tyr-140, Pro-178, Leu-205, Glu-208, Arg-252, and Tyr-253). Maybe these amino acids are important for the protein structure and mutation of one of them does not have such a big effect on the overall structure.

**Modules of TRZ1, Dimerization Module**—We mutated the two cysteines (Cys-25 and Cys-40) in TRZ1 to analyze whether these amino acids mediate dimer formation. The presence of both cysteines was not necessary for dimer formation since both cysteine mutants still form dimers. Both cysteines are not conserved between the tRNase Z enzymes (Fig. 1), and the mutation of Cys-40 does not influence the catalytic activity at all. Mutation of Cys-25 only reduced the tRNA processing activity, showing that both cysteines are not important for TRZ1 activity.

5 amino acids could be identified that, if mutated, resulted in loss of dimer formation, His-59, Pro-83, Thr-186, Lys-203, and His-248, probably by disturbing the whole structure of the protein. The alignment in Fig. 1 shows that TRZ1 is the only tRNase Z sequence having a lysine instead of a conserved aspartic acid. Nevertheless, this Lys seemed to be important for the protein since the mutation results in loss of dimer formation.

The deletion of the two potential leucine zippers resulted in almost complete inactivation since no tRNA binding is observed and no processing activity is detectable. Cross-linking experiments showed, how-

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**FIGURE 7. Potential metal binding of TRZ1.** The metal binding center of the *E. coli* TRZ1 homologue ZIPD was only recently proposed (10). Alignment of TRZ1 with ZIPD shows that identical amino acids are found in TRZ1 at the same positions: His-54, His-56, and His-133 could bind metal ion A (mA), Asp-58, His-59, and His-248 could bind metal ion B (mB), and Asp-185 could bridge both metal ions.

**FIGURE 8. Summary of the effect of TRZ1 mutations.** Mutations that result in total loss of catalytic activity (but still form dimers and bind to tRNAs) are marked red, and mutations that result in reduced catalytic activity are shown in brown. Regions and amino acids that are important for tRNA binding are marked blue, and mutations that interfere with dimerization are shown in green. Mutations that do not alter any activity of TRZ1 (dimerization, tRNA binding, and catalysis) are shown in gray.
ever, that dimerization still takes place; thus the two regions deleted are not important for dimerization.

Deletion of the 11 amino acids at the C terminus (del270–280) also prevented dimerization. According to the Bacillus tRNase Z structure, the C terminus was not taking part in dimerization. Thus the deletion might disturb the whole protein structure, resulting in formation of aggregates. The structure just published for the bacterial tRNase Z shows that dimerization occurs via α1, α2, α3, and the β1/β2 loops of each monomer. According to the alignment (Fig. 1), the TRZ1 mutations of Cys-40, His-56, Asp-58, His-59, Gly-62, and Pro-64 are located in the region in the B. subtilis enzyme where the helices α1 (Cys-40) and α2 (His-56, Asp-58, His-59, Gly-62, Pro-64) are located. Of these amino acids, only mutation of His-59 was shown to interfere with dimerization.

The crystal structure of the bacterial tRNase Z confirmed this hypothesis since in this model, one monomer of the homodimer is binding the substrate, whereas the other is performing the catalysis. Thus both monomers are required since they might carry out different functions.

**Analysis of the tRNA Binding Module**—The variants, which form dimers but do not bind tRNA, identify amino acids, which are essential for RNA binding. In our mutational analyses, the following amino acids and regions were found to be involved in RNA binding: Gly-184, del51–60, del149–164, and del200–212 (TABLE ONE and Fig. 1). Schilling et al. (26) showed that the deletion of the exosite (an element outside the active site, which participates in substrate binding) of the E. coli Zn2P enzyme (Figs. 1 and 5) results in loss of RNA binding. Our deletion mutant del149–164 was located in this exosite region and showed the same behavior.

Cocrystallization of the Bacillus tRNase Z was done without the tRNA substrate. However, the authors superimposed in silico the tRNA onto the tRNase Z structure and proposed the following regions to be involved in RNA binding: (all contacts lie in subunit B) the exosite, the loop between β2 and β3, and the loop between α1 and α4 (20) (Fig. 5). Comparison of our results and the E. coli data with the Bacillus structure shows that the exosite and del149–164 are located where the authors proposed RNA binding (20). We identified an additional amino acid (Gly-184) in the Arabidopsis protein that does not lie in the region proposed to be involved in RNA binding by de la Sierra–Gallay et al. (20) but is clearly impaired in tRNA binding (Fig. 5). Thus Gly-184 might be a contact point for the tRNA in subunit A. Otherwise, as de la Sierra–Gallay et al. (20) discuss, it might be that the homodimer undergoes rearrangement upon substrate binding, and thus it might be that Gly-184 in subunit B (β12) turns upward upon binding of the substrate.

**Mutations That Reduce Catalytic Activity**—We were surprised to find one of the amino acids, which we suspected to be involved in dimer formation, to have reduced catalytic activity (Cys-25). This mutant formed dimers and bound to tRNAs but only had reduced tRNA processing activity (33%). Similar behavior was found in mutants G62V, Y140L, P178A, L205I, E208A, R252G, and Y253S, which suggests that these amino acids are somehow involved in catalysis since the respective variants still form dimers and bind to tRNA but process pretRNAs with only reduced activity (20–80% when compared with wild type activity).

The **Metal Binding Motif of TRZ1**—Metallo-β-lactamases bind up to two zinc, iron, or manganese ions (19, 27). For the E. coli tRNase Z, homologue ZiPD, two Zn2+ ions are required for catalysis, and Meyer-Klaucke and co-workers (10) proposed a metal coordination for ZiPD. This metal binding site of ZiPD is similar to the metal coordination sphere of glyoxalase II (10, 28). Similar zinc binding was proposed for the B. subtilis enzyme. If we superimpose the corresponding TRZ1 amino acids onto the E. coli model (Fig. 7), His-54, His-56, and His-133 would bind one metal ion, whereas Asp-58, His-59, and His-248 would bind the second metal ion. Asp-185 would bridge both ions. Mutation of the amino acids implicated in metal binding in TRZ1 are all inactive in processing. Only the mutation of the bridging amino acid Asp-185 resulted in reduced processing activity (7%). If the metal binding center of TRZ1 is indeed the same as for ZiPD and glyoxalase II, the tRNA processing enzymes would have the same metal coordination sphere as the glyoxalase. Interestingly, glyoxalase II can replace zinc by iron or manganese (10). Incubation with chelators showed that metals required for the tRNA processing activity of TRZ1 are manganese or magnesium.

**Potential Catalytic Region**—The histidine motif with the highly conserved motif HXHxDH (HxSHxMDxHxH in TRZ1) is a good candidate for being part of the catalytic domain of TRZ1. Deletion of this motif resulted in loss of tRNA binding and processing activity, whereas dimer formation was still possible. The point mutants made in this region (His-54, His-56, Asp-58, and His-59) were all inactive in processing. Thus the histidine motif seems to be an important part of the catalytic domain, providing 3 of the 7 amino acids required for metal binding. Similar observations have been made with mutations in this motif of the homologous proteins from E. coli (10), T. maritima (29), Saccharomyces cerevisiae (30), and Drosophila melanogaster (31).

**Conclusion**—We mutated 24 amino acids of the tRNase Z enzyme, of which 21 are conserved (Fig. 8). Of these conserved amino acids were totally dispensable for dimerization, tRNA binding, and catalysis (class 1). Another 7 gave only a slight reduction of activity (class 2). 4 of the conserved amino acids analyzed were important for dimerization (class 5), whereas only 1 amino acid was essential for tRNA binding (class 4). 6 amino acids were identified that are required for catalysis (class 3). Deletion variants identified additional regions important for tRNA binding and dimer formation.

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