THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 42, pp. 35440–35447, October 21, 2005

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

Received for publication, June 13, 2005, and in revised form, July 28, 2005 Published, JBC Papers in Press, August 22, 2005, DOI 10.1074/jbc.M506418200

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tRNA 3′ processing is one of the essential steps during tRNA maturation. The tRNA 3′-processing endonuclease tRNase Z was only recently isolated, and its functional domains have not been identified so far. We performed an extensive mutational study to identify amino acids and regions involved in dimerization, tRNA binding, and catalytic activity. 29 deletion and point variants of the tRNase Z enzyme were generated. According to the results obtained, variants can be sorted into five different classes. The first class still had wild type activity in all three respects. Members of the second and third class still formed dimers and bound tRNAs but had reduced catalytic activity (class two) or no catalytic activity (class three). The fourth class still formed dimers but did not bind the tRNA and did not process precursors. Since this class still formed dimers, it seems that the amino acids mutated in these variants are important for RNA binding. The fifth class did not have any activity anymore. Several conserved amino acids could be mutated without or with little loss of activity.

tRNA molecules are essential for protein synthesis, providing the amino acids during translation. They are not directly transcribed as functional molecules but as precursor RNAs, which require several processing steps to generate the functional tRNA molecule. Two of these processing steps are the removal of the additional 5′ and 3′ sequences of the tRNA. Although the removal of the additional 5′ sequence (the 5′ leader) is well understood (1), maturation of the tRNA 3′ end is not as well studied, although a correctly generated tRNA 3′ end is essential for the addition of the CCA triplet and thus for aminoacylation (2).

It has been shown that in *Escherichia coli*, tRNA 3′ maturation is a multistep process involving endo- as well as exonucleases, the final steps being performed by an exonuclease (3). In contrast, *Bacillus subtilis* employs an endonuclease, called tRNase[6] Z (EC 3.1.26.11), which cleaves CCA-less tRNA precursors directly 3′ to the discriminator (4). 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^S enzymes, and the long tRNase Z proteins (with 700–950 amino acids), the tRNase Z^L enzymes. Although the tRNase Z^S proteins are present in all kingdoms, the tRNase Z^L 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^L proteins has high sequence similarity to the tRNase Z^S 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

* This work was supported by the VolkswagenStiftung, Fonds der Chemischen Industrie, and the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[S] The on-line version of this article (available at http://www.jbc.org) contains five supplemental figures.
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[6] The abbreviation used is: tRNase, tRNA 3′ endonuclease.

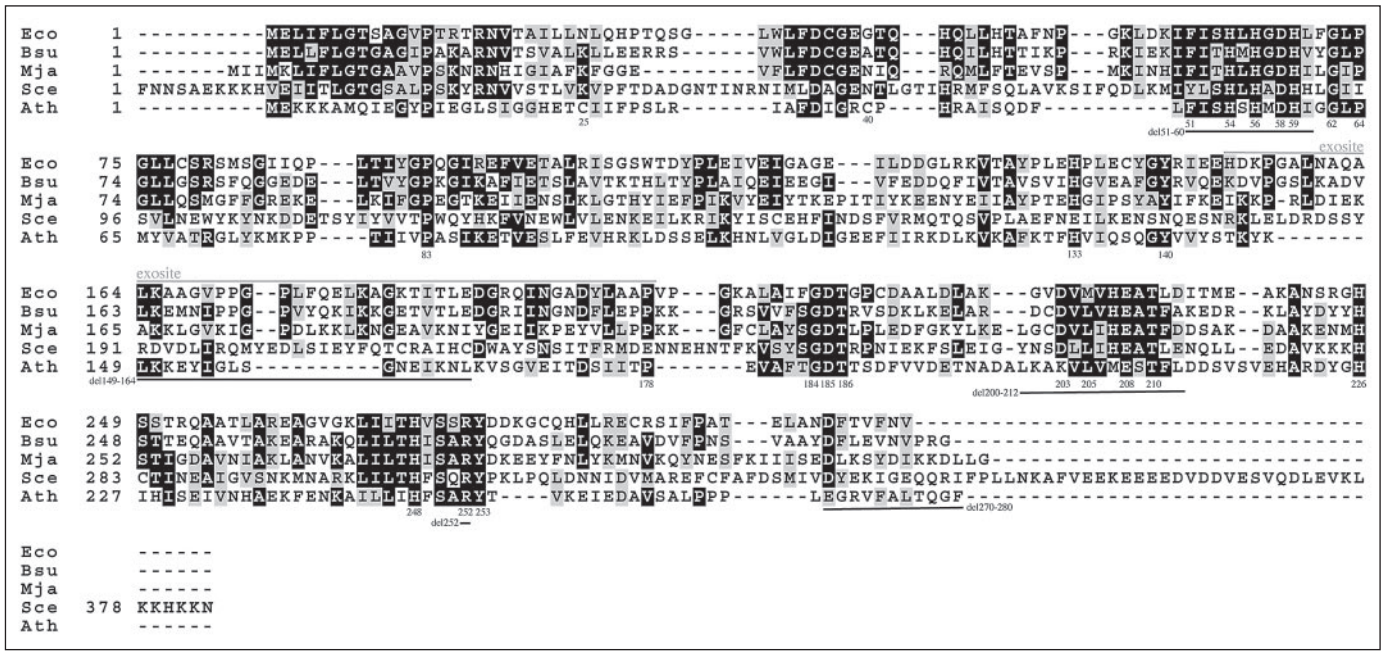

FIGURE 1. **Alignment of tRNase Z protein sequences.** Short tRNase Z protein sequences from *E. coli* (*Eco*) (accession number P0A8V0), *B. subtilis* (*Bsu*) (accession number P54548), *Methanocaldococcus janaschii* (*Mja*) (accession number Q58897), and *A. thaliana* (*Ath*) (accession number Q8LGU7) and the C-terminal part of the long tRNase Z protein from *S. cerevisiae* (*Sce*) (accession number P36159) were aligned to identify conserved regions. Amino acids identical between at least three sequences are *shadowed black*, and amino acids similar between at least three sequences are *shadowed gray*. The position of the point mutations is marked with a *number* below the amino acid changed (the number corresponds to the point mutation number), and deletions are marked with a *line* below the amino acids deleted.

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 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 (both digested with NcoI and XhoI), yielding the pET32a-mutant clones and pET29a-mutant clones, respectively. A list of the variants obtained is shown in TABLE ONE. All constructs were sequenced to confirm the mutations.

Overexpression of Recombinant Proteins—Expression of TRZ1 and TRZ1 variants was done as described previously (5) with the following modification: the strain BL21(DE3)pLys was used for expression (Supplemental Fig. 1). Class 3 variants were separated from GroEL (22), which copurifies during S tag purification, using a MiniQ column, to confirm that loss of processing activity was not due to the presence of GroEL. The column was equilibrated using buffer A (40 mM Tris-HCl, pH 8), a step gradient was applied using buffer B (buffer A with 1 M KCl), and tRNase Z was eluted with 120 mM KCl. The tRNase Z fraction was concentrated and dialyzed against buffer A. All recombinant proteins (wild type and variants) were expressed at the same (low) amounts (100–120 μg of protein/l *E. coli* culture).

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 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 [^{32}P]pCp. 1 fmol of labeled tRNA was heated for 5 min at 80 °C in binding buffer (10 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 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 Vcm^{-1} in TG buffer (10 mM Tris, pH 8, 58 mM glycine). Gels were analyzed by autoradiography.

Substrate Preparation—Precursor $tRNA^{Tyr}$ from *Oenothera berteriana* 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 $MgCl_2$, 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-phenantroline or for 1 h at 4 °C with 10 mM EDTA. Chelators and chelator-metal complexes were subsequently removed by dialysis. *In vitro* proc-

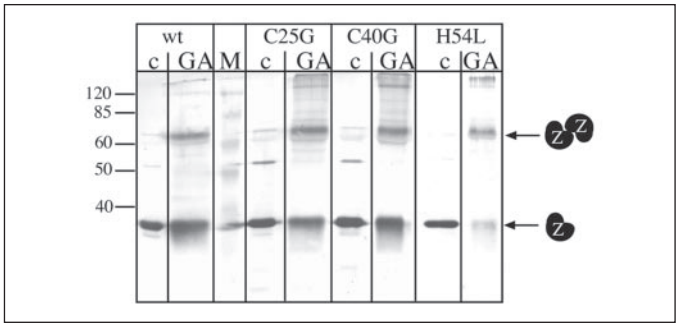

FIGURE 2. **The tRNase Z is a homodimer.** Wild type tRNase Z (TRZ1) and variants C25G, C40G, and H54L were incubated with glutaraldehyde, separated on an SDS-PAGE, and transferred to a western membrane. Monomers and dimers were detected with anti-TRZ1 antibodies. All four proteins form dimers, and the variants even seem to form multimers. *Lanes c* show the control to which no glutaraldehyde was added, and *lanes GA* show the reaction with glutaraldehyde. *Lane wt*, wild type TRZ1; *lanes C25G*, *C40G*, and *H54L*, the respective variants were analyzed. Protein size markers are shown at the *left* in kDa. The TRZ1 monomer and dimer are shown schematically at the *right*.

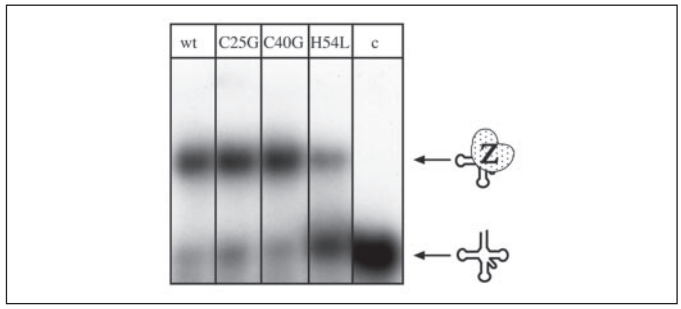

FIGURE 3. **tRNase Z binds to tRNAs.** TRZ1 and variants were incubated with wheat tRNA to analyze whether the proteins bind to tRNA. The reaction was loaded onto a non-denaturing polyacrylamide gel, which was subsequently analyzed by autoradiography. The autoradiograph clearly shows that all four proteins bind to the tRNA (although H54L with lower efficiency). *Lane wt*, wild type protein TRZ1 incubated with tRNA; *lanes C25G, C40G, and H54L*, incubation of variants with tRNA; *lane c*, control without the addition of proteins. The tRNA and the tRNA/protein-complex are shown at the right schematically.

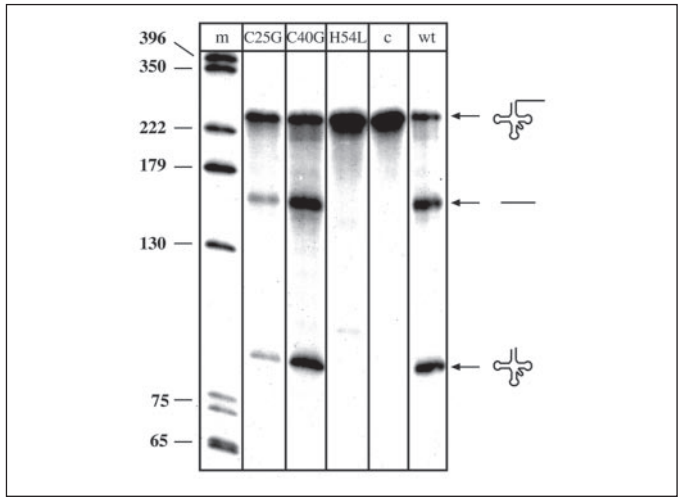

FIGURE 4. **tRNase Z processes precursor tRNAs.** To investigate whether the mutations made interfere with the catalytic activity, proteins were incubated with precursor tRNA molecules. *Lane m*, DNA size marker; *lane c*, control reaction without proteins; *lane wt* (wild type protein TRZ1) and *lanes C25G*, *C40G*, and *H54L*, incubation with the respective proteins. TRZ1 (100% activity, see also TABLE ONE) and C40G (99% when compared with TRZ1) cleave the precursor tRNA efficiently, C25G shows only weak activity (33% when compared with TRZ1), and H54L does not cleave the precursor. DNA size markers are shown in nucleotides at the *left*. Precursor and products are shown schematically at the *right*.

essing reactions were carried out in metal-IVP buffer (40 mM Tris, pH 8.5, 2 mM dithiothreitol), and different metal ions (0.2 mM Mn^{2+}, Fe^{2+}, or Zn^{2+} according to Ref. 18; 2 mM Mg^{2+} according to Ref. 24) were added to analyze their effect on processing. No difference was observed whether the protein was preincubated with metal ions for 3 h at 24 °C or whether the reaction was started (by the addition of the tRNA precursor) immediately after the addition of the metal ions. All buffers were treated with Chelex 100 (Bio-Rad) to remove metal ions.

Determination of Cleavage Efficiency—To determine the cleavage efficiencies, *in vitro* processing products of internally labeled precursors were separated by PAGE gels, which were subsequently dried. Gels were analyzed using a Fuji BAS 1000 instrument (FujiFilm), and processing products were quantified using the software MacBAS (FujiFilm). All experiments were carried out in triplicates, and the resulting data were averaged. The cleavage efficiency of the wild type precursors was set to 100%.

RESULTS

Gel filtration analyses suggested that TRZ1 might be active as 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 $tRNA^{Tyr}$ confirmed the catalytic activity of TRZ1 (Fig. 4).

Rationale for Mutant Selection—To identify important regions and amino acids of TRZ1 for dimerization, RNA binding, and catalysis, we initiated an extensive mutational study of TRZ1. Alignment of tRNase Z protein sequences from different organisms revealed conserved 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*

TABLE ONE

tRNase Z variants

An overview over the mutations made is shown. The Mutation column indicates where the mutation was made; (c) identifies amino acids conserved in at least four of the five tRNase Z sequences aligned in Figure 1. The xlink column results from the cross-link experiments; +, protein forms dimers; o, weak dimerization observed; −, no dimerization observed. The EMSA column results from the electrophoretic mobility shift assays; +, protein binds to tRNA; o, weak binding observed; −, no binding observed. The ivp column results from *in vitro* processing experiments. Wild type activity was set to 100%, all TRZ1 variants were compared to the wild type activity. Processing activity is given in % activity compared to wild type activity. The Class column shows the classification of the mutant. Class 1 has wild type activity (80–100 % compared to wild type); class two has reduced catalytic activity (20–80 %); class three forms dimers, binds tRNA but has no processing activity (below 20 %); class four forms dimers but does not bind tRNA and has no catalytic activity; and class five does not form dimers, does not bind tRNA and has no activity. wt, wild type protein TRZ1.

Mutation	xlink	EMSA	ivp	Class
–	+	+	100	–
Deletion 51–60	o	–	–	4
Deletion 149–164	o	–	–	4
Deletion 200–212	o	–	–	4
Deletion 270–280	Multimers	–	–	5
C25G	+	+	33	2
C40G	+	+	99	1
F51L (c)	+	+	95	1
H54L (c)	+	o	–	3
H56L (c)	o	o	–	3
D58A (c)	+	+	–	3
H59L (c)	Multimers	–	–	5
G62V (c)	+	+	26	2
P64A (c)	+	+	98	1
P83L (c)	Multimers	–	–	5
H133L (c)	+	o	–	3
Y140L (c)	o	+	30	2
P178A (c)	+	+	74	2
G184V (c)	o	–	–	4
D185G (c)	+	+	7	3
T186I (c)	Multimers	–	–	5
K203I (c)	Multimers	–	–	5
L205I (c)	o	+	56	2
E208A (c)	+	+	55	2
T210I (c)	o	+	85	1
H226L (c)	+	+	–	3
H248L (c)	Multimers	–	–	5
R252G (c)	+	+	26	2
Deletion of Arg-252	+	+	–	3
Y253S (c)	+	+	23	2

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. Interestingly, 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 tested, only the addition of Mn^{2+} and Mg^{2+} 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 processing activity. Cross-linking experiments showed that these deletion variants barely form dimers anymore (TABLE ONE and Supplemental Fig. 2), that they were not able to bind to tRNAs 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

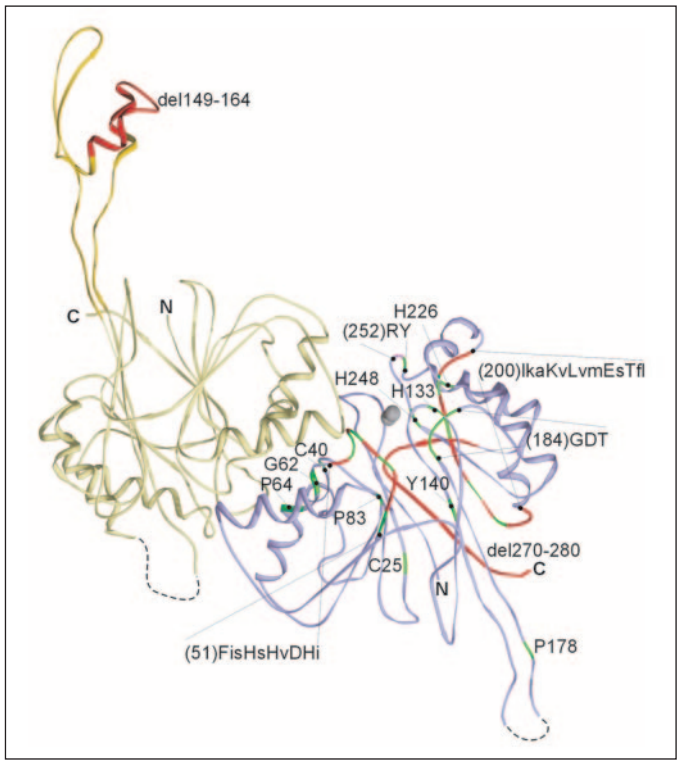

FIGURE 5. **Putative position of the mutations mapped on the *B. subtilis* tRNase Z structure.** A ribbon diagram of the BsuTrz crystal structure (Protein Data Bank code 1Y44) is shown. The homodimer is shown with the different subunits in *blue* and *yellow*, respectively. The zinc atoms are drawn as *gray spheres*, and regions not resolved by crystallography are indicated by a *dotted black line*. The N and C termini are marked. The exosite is drawn in *orange*. The putative positions of the TRZ1 mutations are mapped based on the alignment shown in Fig. 1. Sites with substitutions are colored *green*, and regions that were deleted are *red*. If several amino acids were mutated, only the position of the 1st amino acid is given. *(51)FisHsHvDHi*, deletion 51–60 is shown in red, and point mutants are shown in *green* and in *capital letters* (Phe-51, His-54, His-56, Asp-58, His-59). *(184)GDT*, positions of the three point mutants Gly-184, Asp-185, and Thr-186 are shown. *(200)IkaKvLvmEsTfI*, deletion 200–212 is shown in *red*. Point mutants in this region are shown in *green* and in *capital letters* (Lys-203, Lys-205, Glu-208, Thr-210). *(252)RY*, residue Arg-252, which was substituted and deleted, is colored in *pink*. Residue 253 was mutated.

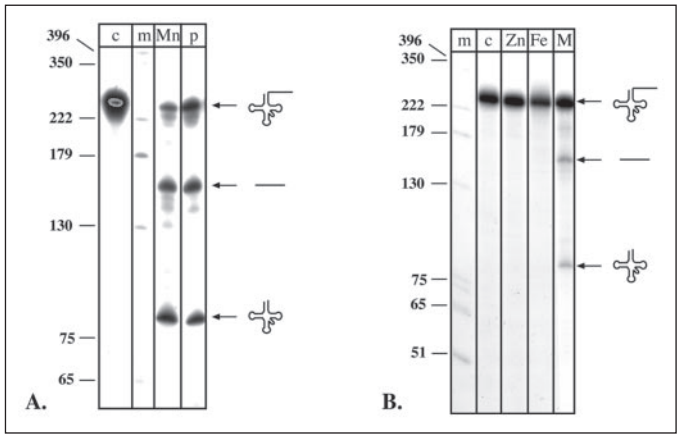

FIGURE 6. **Chelator inhibit processing.** Processing reactions were preincubated with the chelator 1,10-phenantroline as described in "Experimental Procedures." *A*, the addition of Mn^{2+} rescues the *in vitro* processing activity of TRZ1 (*lane Mn*). *Lane p*, Processing reaction without preincubation with 1,10-phenantroline. *B*, Zn^{2+}, Fe^{2+} do not rescue the activity (*lanes Zn* and *Fe*), but the addition of Mg^{2+} does rescue the activity (*lane M*). *Lanes m*, DNA size marker (sizes are indicated in nucleotides at the *left*); *lanes c*, control reactions without the addition of proteins. Precursor and products are shown schematically at the *right*.

does not bind to tRNAs and does not have catalytic activity (TABLE ONE, Supplemental Figs. 2–4). Therefore the 11 amino acids from the C terminus are essential for tRNase Z activity.

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 Have Only Reduced Catalytic Activity (20–80%) (Class Two)—Variants C25G (Figs. 2–4), G62V, Y140L, P178A, L205I, E208A, R252G, and Y253S (Supplemental Figs. 2–4) are still able to form dimers and still bind to 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.

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. This suggests a direct involvement of amino acids His-54, His-56, 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.

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, H133L, and H226L. The addition of imidazole did, however, not rescue the catalytic activity of these mutants (data not shown).

Variants That Do Not Bind or Bind Only Weakly to tRNAs (Class Four)—Several variants (del51–60, del149–164, del200–212, and G184V) were still able to form dimers but did not bind to tRNAs anymore. They also did not process precursor tRNAs (Supplemental Figs. 2–4), suggesting that the mutated amino acid Gly-184 and that the deleted regions are important for RNA binding.

Variants That Do Not Form Dimers (Class Five)—Variants H59L, P83L, T186I, K203I, H248L, and del270–280 did not form dimers anymore but seemed to aggregate since glutaraldehyde cross-linking results in bigger complexes, probably representing multimers. This suggested that the change of these amino acids somehow disturbs the whole protein structure, which leads to complete loss of activity. Supporting this hypothesis is the fact that these variants cannot be separated from the *E. coli* chaperonin GroEL. Neither anion exchange chromatography nor gel filtration analysis (even with the addition of ATP and Mg^{2+}) succeeded in separating these TRZ1 variants from GroEL.

DISCUSSION

Although tRNA 3′ end processing is vital for tRNA maturation and subsequent aminoacylation, little is known at present 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-

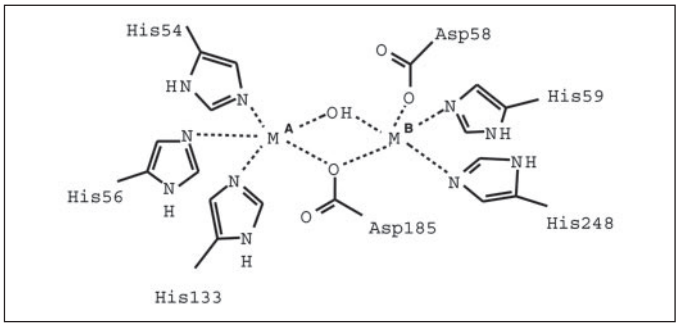

FIGURE 7. **Potential metal binding of TRZ1.** The metal binding center of the *E. coli* TRZ1 homologue ZiPD was only recently proposed (10). Alignment of TRZ1 with ZiPD shows that identical amino acids are found in TRZ1 at the same positions: His-54, His-56, and His-133 could bind metal ion A (*mA*), 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* ZiPD enzyme (Figs. 1 and 5) results in loss of tRNA binding. Our deletion mutant del149–164 was located in this exosite region and showed the same behavior.

Crystallization 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 β4 (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 Zn^{2+} 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 H*X*H*X*DH ($H_{54}SH_{56}MD_{58}H_{59}$ 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). 3 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.

Acknowledgments—We thank Elli Bruckbauer and Ingrid Schleyer for expert technical assistance.

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