Analysis of the functional modules of the tRNA 3’ endonuclease (tRNase Z)

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Running title: tRNase Z variants

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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 dimerisation, 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 has wild type activity in all three respects. Members of the second and third class still form dimers and bind tRNAs but have reduced catalytic activity (class two) or no catalytic activity (class three). The fourth class still forms dimers but does not bind the tRNA and does not process precursors. Since this class still forms dimers, it seems that the amino acids mutated in these variants are important for RNA binding. The fifth class does not have any activity anymore. Several conserved amino acids can 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. While 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 E. 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 Z (E.C. 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). Database analyses showed that TRZ1 homologues are present in organisms from all three kingdoms, Bacteria, Archaea and Eukarya (Figure 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 ZS and the long tRNase Z proteins (with 700 to 950 amino acids), tRNase ZL. Whereas the tRNase ZS proteins are present in all kingdoms, the tRNase ZL enzymes can only be found in eukarya. Both subgroups are part of the same protein family since the C-terminal part of the tRNase ZL proteins has high sequence similarity to the tRNase ZS enzymes. TRZ1 belongs to the family of metal dependent β-lactamases (9), a group of
metalloproteins which 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 (CPSF) (13), SNM1 (also named PSO2) and Artemis (14,15), proteins which are involved in DNA repair (16). Another subgroup consists of cAMP phosphodiesterase enzymes (17), which catalyse 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 *Bacillus subtilis* (20) and *Thermotoga maritima* (21) confirm that the tRNase Z enzymes belong to the family of metal dependent β-lactamase 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 which participates in substrate binding) protrudes from the main protein body pointing towards the solvent.

We are currently analysing the functional modules of the eukaryotic tRNase Z enzyme, TRZ1 from *Arabidopsis thaliana*. It is a short tRNase Z enzyme of 280 amino acids containing two potential leucine zippers (LZ) and a histidine motif that is part of a metallo-β-lactamase motif containing three highly conserved histidine residues. To identify amino acids involved in dimerisation, tRNA binding and catalysis, 24 point mutations were made. In addition several deletion variants were generated which 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 four conserved amino acids to be required for dimerisation and one amino acid and three regions important for tRNA binding. In addition six 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 1. All constructs were sequenced to confirm the mutations.

**Overexpression of recombinant proteins.** Expression of TRZ1 and TRZ1-variants was done as previously described (5) with the following modification: the strain BL21(DE3)pLys was used for expression (Supp. 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, pH8), a step gradient was applied using buffer B (buffer A with 1 M KCl), TRZ1 Z was eluted with 120 mM KCl. The tRNase Z fraction was concentrated and dialysed against buffer A.

All recombinant proteins (wild type and variants) were expressed at the same (low) amounts (100-120 μg protein/1 E. coli culture).

**Cross-linking assay.** For crosslinking assays with glutaraldehyde 1 μg protein was incubated with glutaraldehyde (final concentration 0.05 %) in double distilled water in 10 μl for 30 minutes at room temperature. After addition of 1 μl 1 M lysine, the sample was loaded onto a SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Hybond C, Amersham). TRZ1 monomers and homodimers were detected using a primary rabbit antibody against TRZ1.
Electrophoretic mobility shift assay (EMSA). tRNA from wheat (tRNA isolated from wheat, Type V, SIGMA) was 3’ end labeled with $^{32}$P-pCp. 1 fmol 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 BSA and 5 % glycerol) and allowed to cool down to room temperature. Recombinant TRZ1 (100 ng), PMSF and DTT were added (to a final concentration of 0.5 mM and 1mM, respectively) and the reaction was incubated for 20 minutes at room temperature. Finally, 2 µl 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 analysed 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 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 (DTT)) at 37°C for 30 minutes. To investigate the effect of imidazol upon the reaction, imidazol (1 mM 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 (PAA) gels. Gels were analysed by autoradiography.

Metal dependency of the in vitro processing reaction

To analyse which metal ions are required for the processing reaction, reactions were preincubated for 5 minutes at 37°C with 1 mM and 10 mM 1,10-phenantroline, or for 1 hour at 4°C with 10 mM EDTA. Chelators and chelator-metal complexes were subsequently removed by dialysis. In vitro processing reactions were carried out in metal-ivp buffer (40 mM Tris, pH 8.5; 2 mM DTT) and different metal ions (0.2 mM of Mn$^{2+}$, Fe$^{2+}$ or Zn$^{2+}$ according to (18), 2 mM Mg$^{2+}$ according to (24)) were added to analyse their effect on processing. No difference was observed whether the protein was preincubated with metal ions for 3 hours at 24°C or whether the reaction was started (by addition of the tRNA precursor) immediately after addition of the metal ions. All buffers were treated with Chelex 100 (BioRad) to remove metal ions.

Determination of cleavage efficiency. To determine the cleavage efficiencies, in vitro processing products of internally labelled precursors were separated by PAGE which were subsequently dried. Gels were analysed using a Fuji BAS 1000 instrument (FujiFilm), 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 crosslinking 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 (EMSA) with TRZ1 and all variants.

As shown previously the recombinant tRNase Z TRZ1 from Arabidopsis thaliana cleaves tRNA precursors efficiently in vitro (5). Thus as 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 (Figure 2). EMSA analyses revealed that TRZ1 binds tightly to tRNA molecules but only weakly to the synthetic precursor tRNA (Figure 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 (Figure 4).

Rationale for mutant selection

To identify important regions and amino acids of TRZ1 for dimerisation, 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 which we chose for mutations (Figure 1, Table 1). Motif

3
search software predicted three regions to be a histidine motif and two potential leucine zippers, respectively. Therefore we made three internal deletions: (1) del51-60, which spans the histidine motif, (2) del149-164 and (3) del200-212, which span the two potential leucine zippers (starting and ending with the first and third 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 (Figure 1). Of particular interest were the amino acids from the histidine motif, which are conserved between the metall dependent β-lactamases (H54, H56, D58 an H59) and the two histidines (H133 and H226) which might be involved in metal binding (11). In addition to mutation of conserved amino acids we also mutated three amino acids which are specific for TRZ1. C25 and C40 can only be found in the TRZ1 sequence and could be involved in dimer formation. K203 is also unique for the TRZ1 sequence and is 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 regard of any of the three activities tested (dimerisation, 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 (P83, T186, del270-280) it was not possible to separate GroEL from the TRZ variants. Interestingly these proteins were also not able to form dimers. GroEL does not interfere with dimerisation, since the other proteins dimerize also in the presence of GroEL.

All variants were analysed in respect to their ability to form dimers, to bind to tRNA and to catalyse 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 (Figure 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 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 (Figure 6). Preincubation with 10 mM 1,10-phenantroline and EDTA inhibited the reaction. Of several metal ions tested, only 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 1 and Suppl. Fig. 2-4) resulted in almost complete inactivation of the processing activity. Crosslinking experiments showed that these deletion variants barely form dimers anymore (Table 1 and Suppl. Fig. 2), they were not able to bind to tRNAs anymore and 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 multimers. In addition this variant does not bind to tRNAs and does not have catalytic activity (Table 1, Suppl. Fig. 2-4). Therefore the 11 amino acids from the C-terminus are essential for tRNase Z activity.

**Amino acids which are not essential for activity**

Variants C40G (Figures 2-4), F51L, P64A and T210I (Suppl. Fig. 2-4) showed the same activity or nearly the same activity (80-100%) as the wild type protein, suggesting that amino acids C40, F51, P64 and T210 are not essential for dimerisation, tRNA binding and catalysis. Amino acid C40 is not conserved throughout the tRNase Z proteins and present only in TRZ1 from Arabidopsis, thus it is not too
suprising that mutation of that amino acid does not interfere with activity. But F51, P64 and T210 are conserved in at least four of the five protein sequences aligned in Figure 1, thus it is interesting that these amino acids do not seem to be essential for processing activity.

**Variants which have only reduced catalytic activity (20-80%)**

Variants C25G (Figures 2-4), G62V, Y140L, P178A, L205I, E208A, R252G and Y253S (Suppl. Fig. 2-4) are still able to form dimers and still bind to tRNAs. But they cleave tRNA precursors with reduced activity (20-80% wild type activity, see Table 1). Since these variants all still form dimers and bind to tRNAs, the mutation seems to affect only the catalytic activity.

**Variants which do not have catalytic activity**

Variants H54L (Figures 2-4), H56L, D58A, H133, D185, H226L and delR252 (Suppl. Fig. 2-4) still form dimers and bind to tRNA but are not able to process precursor tRNAs. This suggest a direct involvement of amino acids H54, H56, D58, H133, D185 and H226 in catalysis. The mutation of amino acid R252G only reduces the catalytic activity (down to 26%, see above), deletion of this amino acid however results in total loss of activity.

In the case of ribozyme-mutants, which were not catalytically active anymore, addition of imidazol rescued the catalytic activity (25). To test whether addition of imidazol to the histidine mutants can rescue the catalytic activity we added 1 mM and 10 mM imidazole to the processing reaction with mutants H54L, H56L, H133 and H226L. Addition of imidazol did however not rescue the catalytic activity of these mutants (data not shown).

**Variants which do not bind or bind only weakly to tRNAs**

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 (Suppl. Fig. 2-4). Suggesting that the mutated amino acid G184 and the deleted regions are important for RNA binding.

**Variants which do not form dimers**

Variants H59L, P83L, T186I, K203I, H248L and del270-280 do not form dimers anymore but seem to aggregate since glutaraldehyde crosslinking results in bigger complexes, probably representing multimers. This suggests 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 can not be separated from the E.coli chaperonin GroEL. Neither anion exchange chromatography nor gel filtration analysis (even with addition of ATP and Mg²⁺) 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 Arabidopsis thaliana to identify amino acids and regions important for dimerisation, tRNA binding and catalysis.

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

**Characteristics of the tRNase Z enzyme from Arabidopsis thaliana**

The TRZ1 protein is a homodimer as the homologous proteins from E. coli (18), T. maritima (21) and B. subtilis (20), it tightly binds to tRNAs and is catalysing 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 suprising that the deletion at the C-terminus inactivates the protein since it is a region were...
only few amino acids are conserved between two or three of the aligned protein sequences (Figure 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 \( \beta \) sheet \( \beta 16 \) (Figure 5). Maybe this particular \( \beta 16 \) is important to stabilize the structure in that region since it is in proximity to the proposed catalytic center in subunit A (20).

**Changes which do not interfere with processing**

It is quite surprising that three conserved amino acids (F51, P64 and T210) can be mutated without much reduction of the processing activity (variants have still 80-100% activity compared to the wild type activity). The change of additional seven conserved amino acids results in only reduced catalytic activity (G62, Y140, P178, L205, E208, R252, Y253). 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: dimerisation module**

We mutated the two cysteins (C25 and C40) in TRZ1 to analyse whether these amino acids mediate dimer formation. The presence of both cysteines is not necessary for dimer formation since both cysteine mutants still form dimers. Both cysteins are not conserved between the tRNase Z enzymes (Figure 1) and the mutation of C40 does not influence the catalytic activity at all. Mutation of C25 only reduces the tRNA processing activity showing that both cysteines are not important for TRZ1 activity.

Five amino acids could be identified, which if mutated, result in loss of dimer formation: H59, P83, T186, K203 and H248 probably by disturbing the whole structure of the protein. The alignment in Figure 1 shows that TRZ1 is the only tRNase Z sequence having a lysine instead of a conserved aspartic acid. Nevertheless this K seems to be important for the protein, since the mutation results in loss of dimer formation.

The deletion of the two potential leucine zipper results in almost complete inactivation since no tRNA binding is observed and no processing activity is detectable. Crosslinking experiments show however that dimerisation still takes place, thus the two regions deleted are not important for dimerisation.

Deletion of the 11 amino acids at the C-terminus (del270-280) also prevents dimerisation. According to the *Bacillus* tRNase Z structure the C-terminus is not taking part in dimerisation. 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 dimerisation occurs via \( \alpha 1, \alpha 2, \alpha 3 \) and the \( \beta 1/\beta 2 \) loops of each monomer. According to the alignment (Figure 1) the TRZ1 mutations C40, H56, D58, H59, G62, P64 are located in the region where in the *B. subtilis* enzyme the helices \( \alpha 1 \) (C40) and \( \alpha 2 \) (H56, D58, H59, G62, P64) are located. Of these amino acid only mutation of H59 was shown to interfere with dimerisation.

The crystal structure of the bacterial tRNase Z confirms this hypothesis since in this model one monomer of the homodimer is binding the substrate while 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: G184, del51-60, del149-164 and del200-212 (Table 1 and Figure 1). Schilling et al. showed that the deletion of the exosite (an element outside the active site which participates in substrate binding) of the *E. coli* ZiPD enzyme (Figures 1,5), results in loss of tRNA binding (26). Our deletion mutant del149-164 is located in this exosite region and shows the same behaviour.

Crystallisation of the *Bacillus* tRNase Z was done without the tRNA substrate. But 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 \( \beta 2 \) and \( \beta 3 \), the loop between \( \alpha 1 \) and \( \beta 4 \) (Figure 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 (G184) in the *Arabidopsis* protein which does not lie in the
region proposed to be involved in RNA binding by de la Sierra-Gallay et al. (Figure 5) (20), but is clearly impaired in tRNA binding. Thus G184 might be a contact point for the tRNA in subunit A. Or as de la Sierra-Gallay et al. discuss it might be that the homodimer undergoes rearrangement upon substrate binding and thus it might be that G184 in subunit B (β12) turns upward upon binding of the substrate.

**Mutations which 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 (C25). This mutant forms dimers and binds to tRNAs, but only has reduced tRNA processing activity (33%). Similar behaviour 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 pre-tRNAs with only reduced activity (20-80% compared to 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 homolog ZiPD two Zn$^{2+}$ ions are required for catalysis and Meyer-Klaucke and his coworkers proposed a metal coordination for ZiPD (10). This metal binding site of ZiPD is similar to the metal coordination sphere of glyoxalase II (GlxII) (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 (Figure 7), His54, His56 and His133 would bind one metal ion, while Asp58, His59 and His248 would bind the second metal ion. Asp185 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 Asp185 results 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 glyoxylase. Interestingly glyoxalase II can replace zinc by iron or manganese (10). Incubation with chelators shows 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 (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 results in loss of tRNA binding and processing activity, while dimer formation is still possible. The point mutants made in this region (H54, H56, D58 and H59) are all inactive in processing. Thus the histidine motif seems to be an important part of the catalytic domain, providing three of the seven amino acids required for metal binding.

Similar observation have been made with mutations in this motif of the homologous proteins from *E. coli* (10), *T. maritima* (29), *S. cerevisiae* (30) and *D. melanogaster* (31).

**Conclusion**

We mutated 24 amino acids of the tRNase Z enzyme, of which 21 are conserved (Figure 8). Three of these conserved amino acids are totally dispensible for dimerisation, tRNA binding and catalysis (class 1). Another seven give only a slight reduction of activity (class 2). Four of the conserved amino acids analysed are important for dimerisation (class 5), while only one amino acid is essential for tRNA binding (class 4). Six amino acids were identified which are required for catalysis (class 3). Deletion variants identified additional regions important for tRNA binding and dimer formation.

**Acknowledgements**

We would like to thank Elli Bruckbauer and Ingrid Schleyer for expert technical assistance. This work was supported by the VolkswagenStiftung, Fonds der Chemischen Industrie and the DFG.
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Table 1

**tRNase Z variants.** Overview over the mutations made, wt: wild type protein TRZ1. Column mutation 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. Column xlink: results from the crosslink experiments, +: protein forms dimers, o: weak dimerisation observed, -: no dimerisation observed. Column EMSA: results from the electrophoretic mobility shift assays, +: protein binds to tRNA, o: weak binding observed, -: no binding observed. Column ivp: 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. Column class: 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.

**Figure 1**

**Alignment of tRNase Z protein sequences.** Short tRNase Z protein sequences from *Escherichia coli* (Eco) (acc. no. P0A8V0), *Bacillus subtilis* (Bsu) (acc. no. P54548), *Methanocaldococcus janaschii* (Mja) (acc. no. Q58897) and *Arabidopsis thaliana* (Ath) (acc. no. Q6LQ7) and the C-terminal part of the long tRNase Z protein from *Saccharomyces cerevisiae* (See) (acc. no. P36159) were aligned to identify conserved regions. Amino acids identical between at least three sequences are shadowed black, amino acids similar between at least three sequences are shadowed grey. 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.

**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, the variants even seem to form multimers. Lanes c show the control to which no glutaraldehyde was added, lanes GA show the reaction with glutaraldehyde. Lane wt: wild type TRZ1, lanes C25, C40 and H54: the respective variants were analysed. A protein size marker is shown at the left in kDa. 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 analyse whether the proteins bind to tRNA. The reaction was loaded onto a non denaturing polyacrylamide gel, which was subsequently analysed 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 addition of proteins. The tRNA and the tRNA/protein-complex are shown schematically at the right.

**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), C25G, C40G and H54L: incubation with the respective proteins. TRZ1 (100% activity, see also Table 1) and C40G (99% compared to TRZ1) cleave the precursor tRNA efficiently, C25G shows only weak activity (33% compared to TRZ1) and H54L does not cleave the precursor. A DNA size marker is shown in nucleotides at the left. Precursor and products are shown schematically at the right.
Figure 5

**Putative position of the mutations mapped on the B. subtilis tRNase Z structure.** Ribbon diagramm of the BsuTrz crystal structure (pdb code 1Y44). The homodimer is shown with the different subunits in blue and yellow, respectively. The zinc atoms are drawn as grey spheres and regions not resolved by crystallography are indicated by a dotted black line. The N- and C-terminus is marked. The exosite is drawn orange. The putative positions of the TRZ1 mutations are mapped based on the alignment shown in Figure 1. Sites with substitutions are colored green, regions that were deleted are red. If several amino acids were mutated only the position of the first amino acid is given: (51) FisHsHvDHi: deletion 51-60 is shown in red, point mutants are shown in green (F51, H54, H56, D58, H59). (184)GDT: positions of the three point mutants G184, D185 and T186 are shown. (200)lkaKvLvmEsTfl: deletion 200-212 is shown in red. Point mutants in this region are shown in green (K203, L205, E208, T210). (252)RY: Residue R252 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-phenanthroline as described in "Experimental Procedures". A. Addition of Mn$^{2+}$ rescues the *in vitro* processing activity of TRZ1 (lane Mn). Lane p: Processing reaction without preincubation with 1,10-phenanthroline. B. Zn$^{2+}$, Fe$^{2+}$ do not rescue the activity (lanes Zn and Fe), but 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 addition of proteins. Precursor and products are shown schematically at the right.

Figure 7

**Potential metal binding of TRZ1.** The metal binding center of the *E. coli* TRZ1 homolog ZiPD was only recently proposed (10). Alignment of TRZ1 with ZiPD shows that identical amino acids are found in TRZ1 at the same positions: His54, His56 and His133 could bind metal ion A (mA), Asp58, His59 and His248 could bind metal ion B (mB) and Asp185 could bridge both metal ions.

Figure 8

**Summary of the effect of TRZ1 mutations.** Mutations which result in total loss of catalytic activity (but still form dimers and bind to tRNAs) are marked red, mutations which result in reduced catalytic activity are shown in brown. Regions and amino acids important for tRNA binding are marked blue and mutations which interfere with dimerisation are shown in green. Mutations which do not alter any activity of TRZ1 (dimerisation, tRNA binding and catalysis) are shown in grey.
Supplemental data:

Supplementary Figure 1: SDS gels of TRZ1 and variants
Supplementary Figure 2: Crosslinking of TRZ1 and variants
Supplementary Figure 3: EMSA of TRZ1 and variants
Supplementary Figure 4: In vitro processing experiments of TRZ1 and variants
Supplementary Figure 5: Determination of cleavage efficiencies for the in vitro processing experiments of TRZ1 and variants.
## Overview over tRNase Z variants and their activities

| 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 R252| +     | +    | -   | 3     |
| Y253S (c)       | +     | +    | 23  | 2     |

### Table 1
Späth et al.
| wt c | GA | M c | GA | c | GA | c | GA |
|------|----|-----|----|---|----|---|----|

Figure 2
Späth et al.
|     | wt | C25G | C40G | H54L | c  |
|-----|----|------|------|------|----|
|     |    |      |      |      |    |

Figure 3
Späth et al.
Figure 5
Vögel et al.
Figure 6
Späth et al.
Supplementary Figure 1: SDS gels of TRZ1 variants. All proteins were separated on 10% SDS gels. GroEL (upper protein of approximately 66 kDa) copurifies with the TRZ variants during S protein purification but does not interfere with TRZ1 activity and can be removed using an anion exchange column. Lanes m: protein size marker. Variant K203 contains a single amino acid change (K203I) which results in a faster migration in the SDS gel. 50-100 ng of each protein was loaded and gels were silver stained.
Supplementary Figure 2: Crosslinking of TRZ1 variants. All proteins were crosslinked as described in material and methods. Monomers and dimers of TRZ1 are shown schematically at the sides. Lanes c: control reaction without glutaraldehyde, lanes GA: reaction with glutaraldehyde, lanes m: protein size marker. All SDS gels were 10 % polyacrylamide gels except the one marked with 7.5% (which is a 7.5 % polyacrylamide gel). TRZ variant K203 runs faster than the wild type protein although only a single amino acid is changed. Some proteins show reproducibly only weak crosslinking bands. That could be due to the fact that only part of the proteins in the fraction is folded correctly and thus able to form dimers.
Supplementary Figure 3: **EMSA of TRZ1 variants.** All variants were incubated with tRNA as described in material and methods and subsequently loaded onto non denaturing polyacrylamide gels. The tRNA and the tRNA-protein complex are shown schematically at the right. Lanes wt: wild type protein, lanes c: control reaction without addition of protein.
Supplementary Figure 4: *In vitro* processing experiments of TRZ1 variants. All proteins were incubated with precursor tRNA substrates as described in material and methods. *In vitro* processing with variants D185 and R252 generates the expected products, tRNA and trailer and additional processing products. Lanes c: control reaction without proteins, lanes m: DNA size marker, lanes wt: incubation with wild type protein. The position of the two processing products, tRNA and 3’ trailer are indicated with an arrow.
Supplementary Figure 5: **Determination of cleavage efficiencies for the in vitro processing experiments of TRZ1 and variants.** Cleavage efficiency of the wild type protein TRZ1 was set to 100%. All experiments were carried out three times and the data were averaged, error bars are indicated.
Analysis of the functional modules of the tRNA 3' endonuclease (tRNase Z)
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Simone Aymanns, Jamel Nezzar and Anita Marchfelder

J. Biol. Chem. published online August 22, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M506418200

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