Substitutions in conserved regions preceding and within the linker affect activity and flexibility of tRNase Z\(^L\), the long form of tRNase Z

Makenzie Saoura\(^{aa}\), Kyla Pinnock\(^{aab}\), Maria Pujantell-Graell\(^{abc}\), Louis Levinger\(^{aad}\)*

York College of The City University of New York, Jamaica, New York, United States of America

\(^a\) Current address: Department of Biology, York College/CUNY, Jamaica, New York, United States of America
\(^b\) Current address: College of Medicine, SUNY Health Sciences Center at Brooklyn, Brooklyn, New York, United States of America
\(^c\) Current address: AIDS Research Institute - IrsiCaixa and Health Research, Institute Germans Trias I Pujol (IGTP), Hospital Germans Trias I Pujol, Universitat Autonoma de Barcelona, Badalona, Spain
\(^d\) Current address: PhD Programs in Biochemistry and Molecular, Cellular and Developmental Biology, CUNY, Jamaica, New York, United States of America

* llevinger@york.cuny.edu

Abstract

The enzyme tRNase Z, a member of the metallo-β-lactamase family, endonucleolytically removes 3' trailers from precursor tRNAs, preparing them for CCA addition and aminoacylation. The short form of tRNase Z, tRNase Z\(^S\), functions as a homodimer and is found in all prokaryotes and some eukaryotes. The long form, tRNase Z\(^L\), related to tRNase Z\(^S\) through tandem duplication and found only in eukaryotes, possesses ~2,000-fold greater catalytic efficiency than tRNase Z\(^S\). tRNase Z\(^L\) consists of related but diverged amino and carboxy domains connected by a flexible linker (also referred to as a flexible tether) and functions as a monomer. The amino domain retains the flexible arm responsible for substrate recognition and binding while the carboxy domain retains the active site. The linker region was explored by Ala-scanning through two conserved regions of \(D.\ melanogaster\) tRNase Z: N\(_{dom}\)-T\(_{prox}\), located at the carboxy end of the amino domain proximal to the linker, and T\(_{flex}\), a flexible site in the linker. Periodic substitutions in a hydrophobic patch (F\(_{329}\) and L\(_{332}\)) at the carboxy end of N\(_{dom}\)-T\(_{prox}\) show 2,700 and 670-fold impairment relative to wild type, respectively, accompanied by reduced linker flexibility at N-T inside the N\(_{dom}\)-linker boundary. The Ala substitution for N\(_{378}\) in the T\(_{flex}\) region has 10-fold higher catalytic efficiency than wild type and locally decreased flexibility, while the Ala substitution at R\(_{382}\) reduces catalytic efficiency ~50-fold. These changes in pre-tRNA processing kinetics and protein flexibility are interpreted in light of a recent crystal structure for \(S.\ cerevisiae\) tRNase Z, suggesting transmission of local changes in hydrophobicity into the skeleton of the amino domain.
Introduction

Transfer RNA (tRNA) is central to translation [1]. Sequencing of the first tRNAs established a canonical secondary structure (cloverleaf) which arises from intramolecular base pairing, and a conserved L-shaped tertiary structure. D—T loop pairing forms the elbow, with the antico- don and acceptor stem at opposite ends. CCA at the 3’ end is universally conserved. C₇₄₋₇₅ of P-site tRNA H-bond with large subunit rRNA, positioning it for peptidyl transfer; the 2’OH of A₇₆ in the peptidyl tRNA participates critically in catalysis.

tRNAs are transcribed as precursors and processed by endonucleolytic removal of a 5’ leader by RNase P, first characterized as a ribozyme and later shown to be a protein-only enzyme in mitochondria and chloroplasts (for reviews, see [2], [3]). Some tRNAs are transcribed with introns that are removed by splicing and all tRNAs undergo extensive post-transcriptional nucleoside modification. The 3’ trailer is removed by a combination of endo- and exonucleases in E. coli, in which -CCA₇₆ is transcriptionally encoded. In eukaryotes, -CCA₇₆ is not transcriptionally encoded; CCA-addition is thus required in eukaryotic nuclei and plastids. tRNase Z provides the principal mechanism for endonucleolytic removal of eukaryotic 3’ trailers, leaving the discriminator base (N₇₃) with a 3’-OH ready for CCA addition. This pathway may be complemented by exonucleases in S. cerevisiae ([4], [5]).

The tRNase Z function and a gene encoding the enzyme are widely conserved [6]. A short and long form (tRNase Z⁵ and tRNase Z⁷, respectively) both endonucleolytically cleave pre-tRNA 3’ end trailers, however the two forms are unevenly distributed among the domains of life. Bacteria and archaea exclusively possess tRNase Z⁵. While tRNase Z⁵ is found in some eukaryotes, tRNase Z⁷ is more widespread (for example, tRNase Z⁵ is absent from S. cerevisiae, C. elegans and D. melanogaster).

tRNase Z is a member of the β-lactamase family of metal-dependent hydrolases, characterized by an αβ/βα sandwich fold with the active site located at the interface between the domains [7]. Motifs I–V are conserved, including seven residues (His and Asp) that coordinate binding of two Zn²⁺ ions which direct H₂O in general in-line acid-base catalysis, four of them in the signature His cluster (HxHxDH; Motif II).

tRNase Z⁵ functions as a homodimer of identical subunits. In addition to Motifs I–V, a unique flexible arm [8], [9] protrudes from the globular core of tRNase Z and binds the elbow of tRNA, directing the acceptor stem including the scissile bond into the active site of the enzyme. The flexible arm in subunit 1 thus positions the 3’ end of the substrate in the active site of subunit 2.

Sequence and structural studies show that tRNase Z⁷ emerged as a tandem duplication of tRNase Z⁵ with subsequent divergence of the amino and carboxy domains (first suggested by [10] and subsequently supported by numerous studies, reviewed in [11]). The amino domain retained the flexible arm but lost the key His and Asp residues from the catalytically important motifs otherwise related by sequence, while the carboxy domain retained functional motifs required for catalysis and lost the flexible arm. The resulting enzyme is better adapted for pre-tRNA 3’ end processing, based on ~2,000-fold higher catalytic efficiency of H. sapiens tRNase Z⁷ than that of tRNase Z⁵ [12]. tRNase Z⁷ is a monomer in solution based on size exclusion chromatography [13] and in the recently solved crystal structure of S. cerevisiae tRNase Z [11] (a tRNase Z⁷).

A 62–85 residue flexible linker joins the conserved, relatively stable amino and carboxy domains in tRNase Z⁷ [13]. The boundary between the linker and the carboxy domain is delineated by homology between the carboxy domain of tRNase Z⁷ and the amino end sequence of tRNase Z⁵. Interestingly, the linker spans the protein surface like a flexible strap [11]; the interface between the amino and carboxy domains of tRNase Z⁷ is much like the dimer interface of tRNase Z⁵.
Within the amino domain of tRNase Z⁴, sequences align with the carboxy domain of tRNase Z⁵ and with tRNase Z⁸ up to and including the flexible arm. In the second half of the amino domain, homology blocks identifiable in tRNase Z⁴’s are less clearly related to sequences in the carboxy domain.

We used previously developed methods ([14]; [13]) to investigate the function and flexibility in regions preceding and within the linker. Ala scans (substitution of alanine for each wild type residue) with processing kinetics were performed followed by flexibility analysis of selected variants. Results were interpreted based on local changes in hydrophobicity in light of the newly available S. cerevisiae tRNase Z structure [11].

Methods

Structure modeling

Secondary structure prediction was performed using PsiPred. Hydropathy plots were obtained using the Wolfenden subprogram [15] in ExPASy. The 1st inframe methionine (MYLV...) of D. melanogaster tRNase Z (NCBI NP_724916.1) and the following 19 residues are interpreted to be a mitochondrial targeting sequence [16] and the nuclear form (presented here) is numbered from the 2nd inframe methionine (...MAAT...). The recently published S. cerevisiae tRNase Z structure [11] was interpreted using PyMOL [17].

Ala scanning mutagenesis

Conserved regions were selected for Ala scanning mutagenesis, one just before the flexible linker and two within the linker. N_{don}, T_{prox} consists of 19 residues in the last homology block in the amino domain on the amino side of the linker (H315 – G333). T_{flex} consists of 9 residues from the most flexible conserved internal region of the linker (M376 – R384). The PEEY region, glutamate rich and less conserved, consists of 9 residues further toward the carboxy end of the linker (P397 – H405). These 37 residues were individually substituted with alanine by replacing the wild type codon at each position with a GCC triplet using A, B amplification and A-B segment joining by PCR and overlap extension PCR, as previously described [18]. ~40-mer oligonucleotides were typically used with the 1, 2 or 3 nt substitution in the middle and with a GC-rich cluster at the 3’ end for stability of primer annealing. The AflII site (nt 1077–1082) subcloning forward primer combined with the reverse mutagenesis primer were used to amplify the A segment using a wild type template. The coding strand (forward) mutagenesis primer combined with the SacI site (nt # 1527–1532) subcloning reverse primer were used to amplify the B segment. A and B segments were gel purified and joined by overlap extension and amplification using the AflII forward and SacI reverse primers. Joined segments were gel purified, recovered, double digested, recovered, and ligated into the AflII-SacI digested vector from which the 454 bp wild type segment had been removed. Plasmids that passed the RE screen were sequenced (Macrogen) to confirm presence of each intended GCC codon and absence of any other sequence changes. The FastBacHT (Invitrogen) transfer vectors with variant tRNase Z cDNAs were transposed into bacmids using DH10Bac (Invitrogen). Large true white colonies produced by successful transformation and transposition were selected for bacmid DNA isolation and transfection into insect Sf9 cells using Cellfectin 2 reagent (Invitrogen).

Baculovirus expression and affinity purification

Amplified baculoviruses with variant D. melanogaster tRNase Z cDNAs were used to infect insect Sf9 cells for 72 h using Hyclone SFX insect cell medium supplemented with 0.5% FBS to minimize degradation of recombinant proteins by endogenous proteases. Cells were lysed
with NP40, expressed proteins were affinity purified using Ni-NTA Sepharose (Qiagen) and the 6XHis tag was cleaved overnight at 4°C with AcTEV protease as previously described [18].

**tRNase Z reaction kinetics**

Nuclear encoded pre-tRNA\^{38} transcript was prepared with T7 RNA polymerase and cleaved using a cis-acting hammerhead leaving a 5’-OH at +1 of the tRNA as previously described [19]. Kinasing with γ-32P-ATP by polynucleotide kinase was performed at +1 of the tRNAs, followed by gel purification and recovery. The processing reaction buffer (PB) consisted of 25 mM Tris-Cl pH 7.2, 2.5 mM MgCl\textsubscript{2}, 1 mM freshly prepared DTT, and 100 μg/ml BSA. Unlabeled substrate concentration was varied over a range of 4–100 nM with a fixed trace amount of 5’-labeled substrate. tRNase Z stocks were adjusted to 25 μM before use from which a dilution series was prepared. Analytical lanes were run with known concentration standards for both input tRNase Z and unlabeled tRNA and the enzyme and unlabeled substrate concentrations used in each experiment was corrected accordingly. Reactions at 28°C were sampled after 5, 10 and 15 min, and quenched with formamide-marker dye mix on ice. Electrophoresis of the samples was carried out on a 6% polyacrylamide gel containing 8 M urea. Gels were dried and exposed overnight using a phosphor screen, which was scanned using a Typhoon 9410 imager and analyzed with IQTL v8.1. Each lane trace yielded a % product and the time course results were converted to % product/min using Excel, equivalent to 0.01 X V/[S], then converted to V X 10\textsuperscript{-11} M/min by multiplying by nM [S], and further analyzed using the single ligand binding function in SigmaPlot. \(k_{cat}\) was obtained by dividing \(V_{max}\) by [E]. Concentration of each variant enzyme was adjusted as necessary depending on the impairment factor observed in previous kinetic experiments. The processing experiments with each variant were repeated until acceptable standard errors were achieved.

**Flexibility of wild type and variant tRNase Z analyzed by limited proteolysis and protein electrophoresis**

Wild type and selected variant tRNase Zs were proteolyzed with trypsin at 1 μg/ml in PB at 28°C and reactions were sampled after 0, 3, 10 and 30 min reaction. Limited proteolysis reactions were analyzed on 1D SDS polyacrylamide gels or using a 2D system (BioRad) with isoelectric focusing in 0.75 mm diam 1\textsuperscript{st} dimension tube gels and SDS electrophoresis in the 2\textsuperscript{nd} dimension as previously described [13]. Protein bands and spots were detected by staining with Sypro Orange and scanning with a Typhoon 9410 and quantitated using IQTLv8.1.

**Results**

A local hydropathy plot [15] provides a useful extension to PsiPred for interpretation of tRNase Z structure and flexibility (Fig 1). For example, pronounced hydrophobicity troughs found close to both ends of the protein, typical of globular proteins in aqueous solution, coincide with flexible regions (cf [13]). N-T and T\textsuperscript{flex}, the two most flexible regions in the linker, are also predicted local hydrophobicity troughs.

The carboxy domain of tRNase Z\textsuperscript{L} is homologous to tRNase Z\textsuperscript{S}, including the active site. Similarly, the flexible arm (FA) in tRNase Z\textsuperscript{L} is related to one of the three branches of flexible arms [9], and the sequence that precedes it is also related, in agreement with the evolution of tRNase Z\textsuperscript{S} from a tandem duplication of tRNase Z\textsuperscript{S} followed by divergence of the amino and carboxy domains. Less is known about the flexible linker of tRNase Z\textsuperscript{L}, however. The S. cerevisiae tRNase Z linker closely follows the exterior contours of the protein as it joins the amino and carboxy domains ([11]; Fig 2). A multiple sequence alignment (Fig 2A; see [20]) combined
Fig 1. D. melanogaster tRNase Z primary structure and prediction of secondary structure and hydrophobicity. The amino acid sequence is shown with secondary structure predicted by PsiPred. Rectangles enclose the amino and carboxy domains joined by the flexible linker; dashed lines indicate conserved motifs and black triangles indicate identified trypsin cleavage sites [13] which occur at flexible, hydrophilic regions. Directly above the predicted secondary structure, a hydropathy plot (created with ExPASy using the Wolfenden scale [15]) depicts the relative hydrophobic and hydrophilic character of the corresponding regions, the dashed red line indicating approximate neutrality.

https://doi.org/10.1371/journal.pone.0186277.g001
with the flexibility results ([13]; Fig 1) suggest the most important regions for further investigation of this extrinsic feature of the enzyme.

Little structural information was available on the amino domain and linker until the recent publication of a S. cerevisiae tRNase Z structure [11]. The basic structure of the S. cerevisiae tRNase Z amino domain is an αβ/βα sandwich fold, like that of the carboxy domain. The flexible arm, located between two strands of β twisted sheet, is extruded from the body of the amino domain. The tRNase Z linker spans the globular core of the enzyme like a strap (Fig 2B; [11]). The linker is an adjunct to, not a substitute for, the domain interface between the amino and carboxy domains, which is much like that observed in the tRNase Z6 homodimer ([11]; cf [8]).
N\textsubscript{dom}T\textsubscript{prox} (within the N domain, proximal to the linker) is the last such homology block preceding the linker [19], [13]. Based on the *S. cerevisiae* tRNase Z crystal structure [11], the 1\textsuperscript{st} half of N\textsubscript{dom}T\textsubscript{prox} has little secondary structure, followed by a short \(\beta\) strand and an \(\alpha\) helix (\(\alpha\)8) with high local hydrophobicity. A flexible hydrophilic patch located on the linker side of the amino domain—linker boundary designated N-T, less conserved than N\textsubscript{dom}T\textsubscript{prox}, which in *S. cerevisiae* tRNase Z consists of a short helix followed by a \(\beta\) strand (\(\beta\)13), gives rise to the limited proteolysis species C\textsubscript{dom}1 [13]. Another conserved flexible hydrophilic region designated T\textsubscript{flex}, found ~35 residues within the linker, gives rise to the C\textsubscript{dom}2 family of proteolysis products.

The regions subjected to single residue Ala substitution and kinetic analysis include 19 residues in N\textsubscript{dom}T\textsubscript{prox} and 13 residues in T\textsubscript{flex}. A short sequence further into the linker is characterized by contiguous glutamates (PEEY region). The goal of an Ala scan is to discover residues of sufficient importance that, when replaced by Ala, cause a significant effect on enzyme activity. Such effects were not observed within the PEEY region, which will therefore not be discussed further. The N-T region is generally conserved in location and hydrophilicity but does not align well and was therefore not examined. Once results of processing kinetics were available, flexibility of selected variants with suggestive functional impairments were studied by limited proteolysis with trypsin and protein gel electrophoresis as in [13].

**Substitutions in two bulky hydrophobic N\textsubscript{dom}T\textsubscript{prox} residues close to the N\textsubscript{dom}—linker boundary greatly impair processing and also reduce flexibility in the N-T region**

The Ala scan processing results in the 1\textsuperscript{st} half of N\textsubscript{dom}T\textsubscript{prox}, suggested by PsiPred to be in \(\alpha\)-helix, are unremarkable. Alanine substitutions in two bulky hydrophobics spaced three residues apart close to the carboxy end of N\textsubscript{dom}T\textsubscript{prox}, Phe329Ala and Leu332Ala, strikingly impair processing with impairment factors of 2,700X and 700X relative to wild type (Figs 3 and 4). In the example illustrated (Fig 3), it was necessary to use the Phe329Ala variant at a >1,000-fold higher concentration than wild type enzyme to obtain a comparable series of processing time courses over the range of unlabeled substrate concentrations used in kinetic experiments. These substitutions for bulky hydrophobic residues on the carboxy side of N\textsubscript{dom}T\textsubscript{prox} were selected for further examination for limited proteolysis with trypsin and protein gel electrophoresis (Fig 5 and data not shown). Phe329Ala demonstrated a marked change in the ratio of stable C\textsubscript{dom} products produced upon trypsin cleavage compared to WT tRNase Z (similar results were obtained from Leu332Ala, not shown). The N\textsubscript{dom}T\textsubscript{prox} region is proximal to the preferred trypsin N-T cleavage site at K\textsubscript{348}/K\textsubscript{351} which produces stable C\textsubscript{dom}1species (accompanying schematics at bottom of Fig 5) that differ slightly in size and charge depending on cleavage at clustered basic residues ([14]; Fig 1). The T\textsubscript{flex} site further into the linker at R\textsubscript{384}/K\textsubscript{385} gives rise to the smaller C\textsubscript{dom}2 species. The C\textsubscript{dom}1 to C\textsubscript{dom}2 ratio in WT tRNase Z is 2:1; in the F\textsubscript{329} variant this ratio decreases to 0.33:1, showing that the alanine substitution at F\textsubscript{329} locally reduces N-T site flexibility.

**Effects of T\textsubscript{flex} region substitutions on processing kinetics and local flexibility**

Of the nine T\textsubscript{flex} alanine variants expressed and analyzed with processing kinetics, Arg382Ala at the carboxy end of the T\textsubscript{flex} region showed the greatest impairment factor, an approximately 50X reduced processing efficiency relative to WT tRNase Z (Fig 6). Multiple sequence alignment shows this to be a conserved residue (Fig 2). Asn378Ala, a substitution in a non-conserved residue near the amino boundary of T\textsubscript{flex}, unexpectedly showed a tenfold increase in
processing efficiency (Figs 6 and 7). Additionally, the Asn378Ala substitution markedly reduces local flexibility as shown by limited proteolysis (Fig 8). The Tflex region includes the trypsin cleavage site at R384/K385 which gives rise to the stable Cdom2 species. In WT tRNase Z the spot intensity ratio of Cdom1 to Cdom2 is 1.5:1 (from schematic at bottom of Fig 8, like the

https://doi.org/10.1371/journal.pone.0186277.g003

processing efficiency (Figs 6 and 7). Additionally, the Asn378Ala substitution markedly reduces local flexibility as shown by limited proteolysis (Fig 8). The Tflex region includes the trypsin cleavage site at R384/K385 which gives rise to the stable Cdom2 species. In WT tRNase Z the spot intensity ratio of Cdom1 to Cdom2 is 1.5:1 (from schematic at bottom of Fig 8, like the
Fig 4. Tabulated variant kinetics for the Ala scan through the N$_{\text{dom}}$T$_{\text{prox}}$ region. Means and standard errors of Michaelis-Menten experiments with tRNase Z processing of pre-tRNA$^{\text{Arg}}$. Kinetic parameters re: WT are shown for each variant, calculated using the data from a WT experiment run in tandem the same day and then averaged.

- The form of tRNase Z (WT or Variant),
- The number of times experiment was repeated,
- $K_{\text{cat}}$ Re WT, $K_{M}$ Re WT, $K_{\text{cat}}/K_{M}$ Re WT.

| tRNase Z$^a$ | $n^b$ | $K_{\text{cat}}^c$ | $K_{M}^d$ | $K_{\text{cat}}/K_{M}^e$ |
|-------------|-------|-------------------|-----------|---------------------|
| WT          | 100   | 14.5±2.2          | 2.9±0.33  | 6.1±0.67            |
| H315        | 3     | 0.055±0.010       | 0.59±0.090| 0.10±0.015          |
| R316        | 2     | 1.2±0.74          | 1.2±0.72  | 0.98±0.03           |
| I317        | 3     | 0.98±0.35         | 1.9±0.59  | 0.69±0.29           |
| Q318        | 3     | 2.7±0.86          | 1.5±0.15  | 2.3±0.19            |
| H319        | 2     | 2.4±0.61          | 1.5±0.76  | 1.8±0.47            |
| Q320        | 2     | 0.19±0.031        | 1.5±0.69  | 0.16±0.089          |
| L321        | 3     | 0.05±0.01         | 1.9±0.6   | 0.03±0.01           |
| H322        | 2     | 0.03±0.01         | 0.15±0.12 | 0.42±0.29           |
| Q323        | 3     | 0.050±0.010       | 0.33±0.13 | 0.22±0.081          |
| L324        | 2     | 0.068±0.016       | 0.88±0.29 | 0.12±0.026          |
| A325G       | 2     | 0.35±0.11         | 1.1±0.26  | 0.31±0.10           |
| P326        | 3     | 0.15±0.048        | 0.71±0.22 | 0.21±0.019          |
| Q327        | 2     | 0.27±0.028        | 0.85±0.28 | 0.34±0.079          |
| V328        | 4     | 0.0008±0.0028     | 0.31±0.12 | 0.037±0.0078        |
| F329        | 4     | 0.00018±0.000082  | 0.44±0.059| 0.00037±0.00011     |
| P330        | 5     | 0.040±0.013       | 0.44±0.21 | 0.24±0.10           |
| L331        | 2     | 0.051±0.017       | 0.20±0.060| 0.25±0.016          |
| L332        | 3     | 0.0005±0.00012    | 0.40±0.10 | 0.0015±0.00020      |
| G333        | 4     | 1.2±0.25          | 0.92±0.16 | 1.38±0.24           |

Fig 4. Tabulated variant kinetics for the Ala scan through the N$_{\text{dom}}$T$_{\text{prox}}$ region. Means and standard errors of Michaelis-Menten experiments with tRNase Z processing of pre-tRNA$^{\text{Arg}}$. Kinetic parameters re: WT are shown for each variant, calculated using the data from a WT experiment run in tandem the same day and then averaged. *The form of tRNase Z (WT or Variant), *The number of times experiment was repeated, $K_{\text{cat}}$ Re WT, $K_{M}$ Re WT, $K_{\text{cat}}/K_{M}$ Re WT.

https://doi.org/10.1371/journal.pone.0186277.g004
value obtained in Fig 5). For the Asn378Ala variant this ratio increases to 4:1. Alanine substitution at N\textsubscript{378} thus causes a dramatic decrease in C\textsubscript{dom,2} species seen after trypsin digestion due to a local decrease in flexibility.

A subdomain defined by interior hydrophobicity arises from interactions across the amino domain—Flexible linker boundary

The greatest impairment of tRNase Z activity obtained in the Ala scan through the N\textsubscript{dom,T\textsubscript{prox}} region was observed with substitution of bulky hydrophobics spaced three residues apart (F\textsubscript{329}, L\textsubscript{332}) toward the carboxy end of the region (Figs 3 and 4). The most closely corresponding residues in S. cerevisiae tRNase Z are Y\textsubscript{361} and F\textsubscript{364} in α8 (Fig 2A). If the backbone in this region is α-helical or helix-like (in the D. melanogaster sequence a proline at 330 would be expected to interrupt an α-helical path; Fig 1), these bulky R-groups would point in roughly the same direction, with potential to collaborate in formation of a hydrophobic cluster. Such a local structural subdomain inflated with high hydrophobicity would not be located deep within the protein considering that the flexible linker spans the enzyme surface (Fig 2B).

Based on the recent structure 5MTZ [11], the best candidate hydrophobic partners are I\textsubscript{391} and I\textsubscript{393} in β13 of S. cerevisiae tRNase Z (Fig 9A). α8 in N\textsubscript{dom,T\textsubscript{prox}} is the last homology block at the carboxy end of the amino domain before the start of the flexible linker. β13 is on the carboxy side of the...
N-T hydrophilic patch that marks the amino boundary of the flexible linker, corresponding to the flexible region sensitive to trypsin (K348KTKL) in D. melanogaster tRNase Z which gives rise to the Cdom1 species (Figs 5 and 8, cf [14]). Corresponding hydrophilic residues in S. cerevisiae tRNase Z (E387KDN; blue in Fig 9) are in a short helical element with R-groups facing solvent. Bulky hydrophobic pairing partners for D. melanogaster F329 and L332 in the N-T region of the flexible linker cannot be identified due to imperfect alignment (Fig 2A). Internal subdomains are apparently created by juxtaposition of several bulky hydrophobic R groups shielded from solvent, producing a micellar spherule inflated like a beach ball (Fig 9A). Substitution of either of the identified bulky hydrophobic R groups in Ndom Tprox with the single methyl group of alanine (white in Fig 9B and 9C) leads to hydrophobicity collapse (illustrated with dashed ellipses and arrows). The Y361 side chain -OH also makes a polar contact.

Fig 6. Processing kinetics with wild type tRNase Z and the Tflex variants Asn378Ala and Arg382Ala. The Asn378Ala substitution increases processing efficiency, while the Arg382Ala substitution impairs processing of pre-tRNAArg40. (A) tRNase Z dilutions used in processing experiments were electrophoresed on a 10% polyacrylamide SDS gel and compared to a BSA standard to determine concentrations. (B-D) Kinetic experiments were performed with a constant concentration of 5’ end 32P-labeled pre-tRNAArg40 substrate and varying concentration of unlabeled substrate, from 4.6–114 nM as indicated below gel panels. Reactions were sampled after 5, 10, and 15 minute incubation at 28°C. Wild type enzyme was used at 32.5 pM; N378A enzyme at 5.6 pM, and R382A enzyme at 400 pM (above gel panels). Phosphorimages were obtained using a Typhoon 9410 scanner. % product/minute, equivalent to V/[S], was determined using IQTLv8.1 software. (E-G) Michaelis-Menten plots were created using SigmaPlot, with kinetic parameters displayed on the corresponding graphs.

https://doi.org/10.1371/journal.pone.0186277.g006
with the I₂22 backbone amino group in the β12—α7 loop (not shown); the bulky hydrophobic character of Y₃₆₁ is, however, probably more important than the polarity of its OH group.

### Longer range effects of Nₙ₉₉domTₚrox and Tₜ₉₉flex substitutions on the skeleton of twisted β sheets flanking the flexible arm in the amino domain

Substitutions in both Nₙ₉₉domTₚrox and Tₜ₉₉flex regions exert their effects through interactions with the skeleton of two twisted β sheets that organize the amino domain (Fig 10). Fig 10A shows the full structure of S. cerevisiae tRNase Z (5MTZ) with the amino domain light grey, carboxy domain dark grey, twisted β sheet 15, 14, 1–6 green and 13–7 blue. The hydrophobicity
collapse in $\alpha_8$/$\beta_{13}$ could thus be transmitted into the twisted $\beta$ sheet in the amino domain on the carboxy side of the flexible arm, as suggested by the enlarged view in Fig 10B.

$\beta_{13}$ is a member on one edge of a 7-stranded $\beta$ sheet, in which $H_{397}$ makes backbone H-bonds with the carboxy group of $H_{311}$ and the amino group of $H_{317}$ in $\beta_{12}$ (Fig 10A and 10B). The first four strands from $\beta_{13}$ (13/12/11/10) are parallel; the last two strands (10/9/8/7) are antiparallel, and $\beta_{9,10}$ ascend to and descend from the flexible arm, respectively. The collapse (deflation) of the $\alpha_8$—$\beta_{13}$ spherule arising from substitution of the specific bulky hydrophobic residues in $N_{dom}T_{prox}$ with Ala (Fig 9B and 9C) damages the overall fold of tRNase Z, explaining the 2,700-fold and 700-fold impairment of tRNase Z activity (Figs 3 and 4). This also reduces local flexibility (Fig 5) by occluding the N-T site that produces the $C_{dom}1$ family of spots relative to $T_{flex}$, which produces $C_{dom}2$. In some ways, these long-range effects of changes in internal subdomain hydrophobicity resemble those of the L187A substitution at the flexible arm-hand boundary in the ascending stalk of $D. melanogaster$ tRNase Z, which causes a close to 100-fold impairment in enzyme activity due to increased $K_M$ [14], accompanied by increased flexibility [13].

$T_{flex}$ coincides with a short $\beta$ strand ($\beta_{15}$), one of two short antiparallel $\beta$ strands in the linker ($\beta_{15}$—$\beta_{14}$) which join a twisted sheet ($\beta_{1}$—$\beta_{6}$) on the amino side of the flexible arm through backbone H-bonds between $\beta_{14}$—$\beta_{1}$ (Fig 10C). Concerning the strongest impairment observed in the region with the R382A substitution (Figs 6 and 7), ionized residues on the surface of the protein such as $E_{419}$ and $D_{422}$ in the $S. cerevisiae$ tRNase Z $\beta_{15}$—$\alpha_9$ loop face the polar solvent as expected for $T_{flex}$. Replacement with a small hydrophobic residue could lead to structural eversion in which the substituted residue buries itself in a partially exposed hydrophobic patch, like the effects of the HbS substitution on hemoglobin structure and function.
The reduced flexibility at the $T_{\text{flex}}$ site arising from the N378A substitution (Fig 8) is interpretable in a general way (Fig 10C). The short antiparallel $\beta$ strands $\beta_{14-15}$ in the $S.\ c\text{erevisiae}$ tRNase Z linker are joined through $\beta_{1}$ to an 8-stranded twisted sheet in the order $\beta_{15-14-1-2-3-4-5-6}$ ($\beta_{15-14-1-2-3}$ are antiparallel and $\beta_{3-4-5-6}$ are parallel). The flexible linker clearly

Fig 9. A cluster formed by hydrophobic interactions between residues in $N_{\text{dom}} T_{\text{prox}}$ and N-T. A) The region of $S.\ c\text{erevisiae}$ tRNase Z ([11]; 5MTZ) from $\alpha_{8}$ through $\beta_{13}$ is shown in cartoon using PyMOL. $\alpha_{8}$ and $\beta_{13}$ are in red and a short helical hydrophilic segment preceding $\beta_{13}$ (E387KDN) is in blue with sticks. Key hydrophobic residues in $\alpha_{8}$ and $\beta_{13}$ are shown in ball and stick with dots. (B) $Y_{361}$ is substituted with Alanine (white); (C) F364 is substituted with Alanine. The substitutions in (B, C) model the substitution of the smaller R-group of Alanine for the bulky hydrophobic R-groups in $D.\ melanogaster$ F329 and L332. Dashed ellipse and curved arrow in (B, C) illustrate the collapse from full inflation due to replacement of a bulky hydrophobic residue required to support the regional structure.
Fig 10. Linker interactions with two skeletal β-twisted sheets in the amino domain of tRNase ZL. As illustrated using the crystal structure of *S. cerevisiae* Trz1 [11], short β strands in the flexible linker are incorporated by polar backbone contacts into the two β twisted sheets which provide the structural core of the amino domain tRNase ZL. (A) Overview of the *S. cerevisiae* Trz1 structure (PDB 5MTZ) with the two β twisted sheets in the amino domain highlighted. (B) Isolated view of the β twisted sheet (β7-β13) rotated for optimal
viewing of the β strands. The flexible arm is extruded from the body of tRNase Z between β9 (ascending) and β10 (descending). In the linker, residue H392 in β13 (cyan) forms polar backbone contacts (dashed lines) with H315 and I317 in β12, the neighboring parallel strand. Hydrophobic interactions between bulky hydrophobic residues in α8 of Tprox and β13 of Tflex, shown in Fig 9, are also presented here. (C) View of the second β twisted sheet (β14-15-1-6), showing antiparallel polar backbone contacts between β14, β15, and β1 (dashed lines). N415 in β15 forms backbone polar contacts with T401 in β14. Two residues in β14, V400 and F402, form backbone polar contacts with F4 and F2 in β1, respectively.

https://doi.org/10.1371/journal.pone.0186277.g010

associates here with the twisted β sheet which forms half the skeleton of the amino domain, on the amino side of the flexible arm (Fig 10C).

Based on the alignment in Fig 2A, N415 in S. cerevisae tRNase Z is the most similar residue in position and identity to N378 in D. melanogaster tRNase Z. Replacement of N415 in β15 with a small hydrophobic residue would locally reduce linker flexibility by strengthening skeletal architecture of the amino domain preceding the flexible arm. The reduced linker flexibility arising from the N378A substitution in D. melanogaster tRNase Z (Figs 6 and 7) could thus improve catalytic efficiency by stiffening the skeleton of β structure on the amino side of the flexible arm. Also noteworthy in this regard, the conservative substitution Leu423Phe in H. sapiens tRNase ZL (ELAC2) associated with mitochondrially based cardiac hypertrophy [21] is located at the start of β15.

Conclusion

A biochemical exploration of little-understood regions of D. melanogaster tRNase Z through Ala scanning mutagenesis followed by processing kinetics was aided by analysis of flexibility using limited proteolysis and two-dimensional protein electrophoresis. This approach, informed by interpretation of a recent crystal structure of the S. cerevisiae homolog, uncovered a previously unknown hydrophobic subdomain formed across the amino domain—linker boundary, leading us to suggest that peripheral substitutions affect the skeleton of twisted β sheets in the amino domain on both sides of the flexible arm.

Author Contributions

Conceptualization: Louis Levinger.
Data curation: Kyla Pinnock, Maria Pujantell-Graell, Louis Levinger.
Formal analysis: Makenzie Saoura, Kyla Pinnock, Maria Pujantell-Graell, Louis Levinger.
Funding acquisition: Louis Levinger.
Investigation: Makenzie Saoura, Kyla Pinnock, Maria Pujantell-Graell, Louis Levinger.
Methodology: Makenzie Saoura, Kyla Pinnock, Maria Pujantell-Graell, Louis Levinger.
Project administration: Louis Levinger.
Resources: Louis Levinger.
Supervision: Louis Levinger.
Writing – original draft: Makenzie Saoura, Louis Levinger.
Writing – review & editing: Makenzie Saoura, Louis Levinger.

References

1. Moore PB, Steitz TA. The roles of RNA in the synthesis of protein. Cold Spring Harbor perspectives in biology. 2011 3(11), a003780. https://doi.org/10.1101/cshperspect.a003780 PMID: 21088149
2. Hartmann RK, Gößringer M, Spáth B, Fischer S, Marchfelder A. The making of tRNAs and more—RNase P and tRNase Z. *Progress in molecular biology and translational science* 2009; 85, 319–368. [https://doi.org/10.1016/S0079-6603(08)00808-8 PMID: 19215776](https://doi.org/10.1016/S0079-6603(08)00808-8)

3. Rossmanith W. Of P and Z: mitochondrial RNA processing enzymes. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, 2012; 1819(9), 1017–1026.

4. Chen Y, Beck A, Davenport C, Chen Y, Shattuck D, Tavtigian SV, et al.: Characterization of TRZ1, a yeast homolog of the human candidate prostate cancer susceptibility gene ELAC2 encoding tRNase Z. *RNA* 2005, 20(1), 115–130. [https://doi.org/10.1038/rna.041467.113 PMID: 24249226](https://doi.org/10.1038/rna.041467.113)

5. Skowronek E, Grzchnik P, Spáth B, Marchfelder A, Kufel J. tRNA 3' processing in yeast involves tRNase Z, Rex1, and Rrp6. *RNA*, (2014). 20(1), 115–130. [https://doi.org/10.1261/rna.041467.113 PMID: 24249226](https://doi.org/10.1261/rna.041467.113)

6. Schiffer S, Rosch S, Marchfelder A. Assigning a function to a conserved group of proteins: the tRNA 3'-processing enzymes. *EMBO J.* 2002; 21, 2769–2777. [https://doi.org/10.1093/emboj/21.11.2769 PMID: 12032089](https://doi.org/10.1093/emboj/21.11.2769)

7. Aravind L: An evolutionary classification of the metallo-beta-lactamase fold proteins. *In Silico Biol* 1999; 1:69–91. PMID: 11471246

8. Li de la Sierra-Gallay I, Pellegrini O, Condon C. Structural basis for substrate binding, cleavage and alloster in the tRNA maturase RNase Z. *Nature* 2005; 433, 657–661. [https://doi.org/10.1038/nature03284 PMID: 15654328](https://doi.org/10.1038/nature03284)

9. Schilling O, Spath B, Kostelecky B, Marchfelder A, Meyer-Klaucke W, Vogel A: Exosite modules guide substrate recognition in the ZIPD/ElaC protein family. *J Biol Chem* 2005; 280:17857–17862. [https://doi.org/10.1074/jbc.M500591200 PMID: 15699034](https://doi.org/10.1074/jbc.M500591200)

10. Tavtigian S, Beck A, Camp NJ, Carillo AR, Chen Y, Dayananth P, et al. (2001) A candidate prostate cancer susceptibility gene at chromosome 17p. *Nat. Genet.*, 27, 172–180. [https://doi.org/10.1038/ng84808 PMID: 11175785](https://doi.org/10.1038/ng84808)

11. Ma M, Li de la Sierra-Gallay I, Lazar N, Pellegrini O, Durand D, Marchfelder A, et al. The crystal structure of Trz1, the long form RNase Z from yeast. *Nucleic Acids Res.* 2017; 45(10):6209–6216. [https://doi.org/10.1038/nar.gkx216 PMID: 28379452](https://doi.org/10.1038/nar.gkx216)

12. Yan H, Zareen N, Levinger L. Naturally occurring mutations in human mitochondrial pre-tRNA\(^{\text{Ser(UCN)}}\) can affect the transfer ribonuclease Z cleavage site, processing kinetics, and substrate secondary structure. *Journal of Biological Chemistry* 2005; 280(7):3926–3935.

13. Wilson C, Ramai D, Serjanov D, Lama N, Levinger L, Chang EJ. Stable Domains and Flexible Regions in tRNase ZL, the Long Form of tRNase Z. *PLoS One* 2013; 8(7):e66942.

14. Levinger L, Hopkinson A, Desetty R, Wilson C. Effect of changes in the flexible arm on tRNase Z processing kinetics. *J. Biol. Chem.* 2009; 284:15685–15691. [https://doi.org/10.1074/jbc.M900745200 PMID: 19351879](https://doi.org/10.1074/jbc.M900745200)

15. Radzicka A, Wolfenden R. Comparing the polarities of the amino acids: side-chain distribution coefficients between the vapor phase, cyclohexane, 1-octanol, and neutral aqueous solution. *Biochemistry* 1988; 27(5), 1664–1670.

16. Dubrovsky EB, Dubrovskaya VA, Levinger L, Schiffer S, Marchfelder A. Drosophila RNase Z processes mitochondrial and nuclear pre-tRNA 3' ends in vivo. *Nucleic Acids Res.* 2004; 32, 255–262. [https://doi.org/10.1038/nar.04182 PMID: 14715923](https://doi.org/10.1038/nar.04182)

17. The PyMOL Molecular Graphics System, Schrodinger, LLC.

18. Zareen N, Yan H, Hopkinson A, Levinger L. Residues in the conserved His domain of fruit fly tRNase Z that function in catalysis are not involved in substrate recognition or binding. *J. Mol. Biol.* 2005; 350:189–199. [https://doi.org/10.1016/j.jmb.2005.04.073 PMID: 1595379](https://doi.org/10.1016/j.jmb.2005.04.073)

19. Karkashon S, Hopkinson A, Levinger L. tRNase Z Catalysis and Conserved Residues on the Carboxy Side of the His Cluster. *Biochemistry* 2007; 46:9380–9387. [https://doi.org/10.1021/bi700578v PMID: 17655328](https://doi.org/10.1021/bi700578v)

20. Zhao W, Yu H, Li S, Huang Y. Identification and analysis of candidate fungal RNA 3'-end processing endonucleases tRNase Zs, homologs of the putative prostate cancer susceptibility protein ELAC2. *BMC evolutionary biology* 2010; 10(1), 272.

21. Haack TB, Kopatich R, Freisinger P, Wieland T, Borbach J, Nicholls TJ, et al. ELAC2 mutations cause a mitochondrial RNA processing defect associated with hypertrophic cardiomyopathy. *Am. J. Hum. Genet.* 2013; 93, 211–223. [https://doi.org/10.1016/j.ajhg.2013.06.006 PMID: 23849775](https://doi.org/10.1016/j.ajhg.2013.06.006)