A $tRNA^{Glu}$ that uncouples protein and tetrapyrrole biosynthesis

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Abstract **Glu-tRNA is either bound to elongation factor Tu to enter protein synthesis or is reduced by glutamyl-tRNA reductase (GluTR) in the first step of tetrapyrrole biosynthesis in most bacteria, archaea and in chloroplasts. *Acidithiobacillus ferrooxidans*, a bacterium that synthesizes a vast amount of heme, contains three genes encoding $tRNA^{Glu}$. All $tRNA^{Glu}$ species are substrates in vitro of GluRS1 from *A. ferrooxidans*. Glu-$tRNA^{Glu}_3$, that fulfills the requirements for protein synthesis, is not substrate of GluTR. Therefore, aminoacylation of $tRNA^{Glu}_3$ might contribute to ensure protein synthesis upon high heme demand by an uncoupling of protein and heme biosynthesis.**

Keywords: tRNA specificity; GluRS; GluTR; EF-Tu; Heme

1. Introduction

Glutamyl-tRNA plays two major roles in nature. Apart from the well-documented function in protein synthesis mediated by elongation factor Tu (EF-Tu) [1], Glu-tRNA is also a precursor for the biosynthesis of tetrapyrroles (e.g., heme and chlorophyll) by being a substrate in the first step of the C_5 pathway in plants, archaea and most bacteria [2–4]. Glu-tRNA, formed by glutamyl-tRNA synthetase (GluRS), is converted to δ-aminolevulinic acid (ALA), the universal precursor of tetrapyrroles biosynthesis, in a two-step reaction. First the glutamyl moiety of Glu-tRNA is reduced to glutamate-1-semialdehyde by the action of glutamyl-tRNA reductase (GluTR) [5]. Then the exchange of the amino group from carbon 1 to carbon 2 is catalyzed by the glutamate semialdehyde 1-2 aminomutase to form ALA [6,7]. Thus, Glu-$tRNA^{Glu}$ interacts in a specific way with at least the proteins GluRS, EF-Tu and GluTR.

The identity elements for the specific interaction of $tRNA^{Glu}$ with *Escherichia coli* GluRS have been elucidated. Nucleotides in the acceptor stem and the anticodon as well as a structural element, the augmented D-stem, are important for the specific recognition by GluRS [8]. Conversely, the core structure, rather than specific nucleotides, seems to be the key element for the specific interaction of Glu-$tRNA^{Glu}$ with *E. coli* GluTR [9].

A small fraction of the Glu-tRNA formed by GluRS is routed to tetrapyrrole biosynthesis in *E. coli* and other bacteria. However, the fact that *Acidithiobacillus ferrooxidans*, an acidophilic bacterium that oxidizes ferrous ion as electron donor for respiration, possesses elevated levels of cytochromes (and hence heme) compared to other organisms [10,11], raises the question as to whether this organism uses a specific mechanism to ensure the supply of Glu-tRNA for heme biosynthesis without a detrimental effect on its use in protein biosynthesis.

Two non-discriminating GluRSs enzymes (GluRS1 and GluRS2) with differential specificity for tRNA are part of the *A. ferrooxidans* Glu-tRNA formation system. Strikingly, the partially sequenced genome revealed the existence of two different $tRNA^{Glu}$ (and two $tRNA^{Gln}$) genes whose transcripts were shown to be specific for either one or the other GluRSs. While tRNAs with augmented D-stem are charged by GluRS1, $tRNA^{Gln}_{UUG}$ (3 base pair in the D-stem) is charged exclusively by GluRS2 [12]. The complex organization of the Glu-tRNA formation system in this organism led us to hypothesize that some tRNAs might have a specific role for either protein or heme biosynthesis. To test this hypothesis, we analyzed the *A. ferrooxidans* genome for additional tRNAs that might be substrate of GluRSs. The Glu-tRNA species were tested for binding to EF-Tu and in their ability to be substrates for GluTR as models for their participation in protein or heme biosynthesis. The results led to the conclusion that a newly identified $tRNA^{Glu}_3$ specie supports an uncoupling between protein and heme biosynthesis. This may ensure that the supply of Glu-tRNA for protein biosynthesis is maintained even during times of high heme demand.

2. Materials and methods

2.1. Strain and culture conditions

A. ferrooxidans ATCC 23270 was cultivated at 30 °C in 9 K medium [13] supplemented with Fe_2SO_4 33.3 g/l, at pH 1.6. *E. coli* DH5α or GM 2163 (*dam*-), used for plasmid propagation was grown at 37 °C, in LB medium. *E. coli* BL21 (DE3) was used for overexpression and purification of proteins. When necessary, the LB medium was supplied with 100 μg/ml of ampicillin.

2.2. Identification of genes encoding for $tRNA^{Glu}$ in A. ferrooxidans

tRNAscan-SE (cove only search mode) [14] was used to identify the genes encoding $tRNA^{Glu}$ in the genome of *A. ferrooxidans* (The Institute of Genome Research). The sequence of all identified tRNA genes was confirmed by sequencing of genomic PCR amplification products.

2.3. Reverse transcription and sequencing of tRNA

Total tRNA from *A. ferrooxidans* was prepared using the QIAGEN RNA extraction kit according to the manufacturer's instructions. The 3′ and 5′ ends of tRNAs were ligated for circularization with RNA

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ligase as described previously [15] and reverse transcribed with R-EAf4 primer (5′-tgaacccgtgtccccggct-3′) and reverse transcriptase (Thermoscript). PCR amplification was carried out with Platinum Taq DNA polymerase (Invitrogen) and R-EAf4 and the F-EAf4 (5′-gttagcgccaGCTCCTGG-3′) primers. Sequences of R-EAf4 with lowercase and uppercase correspond to the 3′ and 5′ end of $tRNA_3^{Glu}$, respectively. PCR product was cloned in pCR-2.1 TOPO vector (Invitrogen) and sequenced.

2.4. Preparation and purification of tRNA

In vivo expressed tRNAs were obtained as is described [12]. *A. ferrooxidans* $tRNA^{Glu}$ wild type and variants transcripts were synthesized in vitro by T7 RNA polymerase run-off transcription [16]. DNA fragments carrying the $tRNA^{Glu}$ gene and the T7 RNA promoter were cloned in pUC18. To generate the appropriate 3′-CCA-end of the $tRNA^{Glu}$ transcripts, plasmid DNA carrying the corresponding $tRNA^{Glu}$ gene was digested with *Sap*I ($tRNA_1^{Glu}$), *Bst*NI ($tRNA_2^{Glu}$) or *Alw*I ($tRNA_3^{Glu}$). In vitro transcription reaction was carried out according to Randau et al. [9]. RNA transcripts were subsequently purified by anion exchange chromatography with QIAGEN Tip100 columns according to the manufacturer's instructions.

2.5. Overexpression and purification of recombinant proteins

BLAST search [17] on the genome of *A. ferrooxidans* (ATCC 23270) was used to identify *tuf* and *gtr* (formerly *hemA*) [18] genes. The DNA was PCR amplified with Elongase (Invitrogen) and primers F1tufAf (5′-tgtccaaagggaaatttgagcgg-3′) and R1tufAf (5′-ttattcgaccaccttggagacga-3′) for *tuf* or R1gtrAf (forward: catatgttgcaatcctgtccggcatc) and F1gtrAf (reverse: ctcgagtcatccttcggcgtcactga) for *gtr*, respectively, (underlined are the sequences recognized by *Nde*I and *Xho*I, respectively). An approximately 1.2 kb PCR product was obtained in each case and cloned in pCR-2.1 TOPO vector. *Tuf* gene was subcloned in the *Eco*RI restriction site of pGEX-2T (Amersham Pharmacia Biotech) whereas *gtr* was subcloned in *Xho*I/*Nde*I restriction site of pET15b, generating the recombinant plasmids pGEFAf and pGtrAfET, respectively. pGEFAf, pGtrAfET, pAFgltX1 and pAFgltX2 [12] were transformed in *E. coli* BL21 for overexpression and purification of the corresponding fusion proteins GST · EF-Tu, His_6 · GlutR, GST · GluRS1 and GST · GluRS2, respectively. GST fusion proteins were purified as is described previously [19]. His_6 · GlutR purification was carried out as described in the pET system manual (Novagen).

2.6. Aminoacylation of tRNA

Aminoacylation of tRNA by GluRS1 or GluRS2 was carried out as described [12]. tRNA transcripts were denatured at 90 °C for 3 min and slowly cooled to facilitate RNA folding.

2.7. Formation of EF-Tu · Glu-tRNA · GTP ternary complex

Assay for the formation of EF-Tu · Glu-tRNA · GTP ternary complex is a modification of a previously described procedure [20]. (GST)EF-Tu · GTP binary complex was bound to glutathione–agarose matrix (Sigma). [^{14}C] Glu-tRNA, obtained by charging in vivo expressed tRNA with *A. ferrooxidans* GluRS [12], was added to a final concentration between 74 and 86 nM. Bound [^{14}C] Glu-tRNA was eluted from immobilized GST · EF-Tu and the radioactivity released was measured in a scintillation counter.

2.8. Activity of GluTR

[^{14}C] Glu-$tRNA^{Glu}$ was prepared in a 1 ml reaction mixture according to Schauer et al. [5] using *A. ferroxidans* GluRS1. GluTR activity was measured at 30 °C by the substrate depletion assay [21] with modifications [9] using 200 nM of *A. ferrooxidans* GluTR. The remaining Glu-$tRNA^{Glu}$ was precipitated, washed, and quantified as previously described [22]. Reaction without GluTR for each condition served as control of spontaneous substrate hydrolysis (about 20% of each tested Glu-$tRNA^{Glu}$). The results are the average of at least two determinations.

3. Results

3.1. Identification of A. ferrooxidans genes encoding $tRNA^{Glu}$

Using a bioinformatic approach based on the tRNAScanSE program we identified four different genes encoding $tRNA^{Glu}$ in the *A. ferrooxidans* genome. Two of them ($tRNA_1^{Glu}$ an $tRNA_2^{Glu}$) were previously reported [12]. While $tRNA_3^{Glu}$, one of the newly identified tRNAs, has a scan score (51.21) high enough to be considered a tRNA, $tRNA_4^{Glu}$ might be a pseudogene because of the relatively low score (41.63). Thus, $tRNA_4^{Glu}$ was not considered further. The gene encoding $tRNA_3^{Glu}$ is located within a cluster of 34 other tRNA genes spanning approximately 10 kb of the genome.

Two peculiar features of the D stem of $tRNA_3^{Glu}$ are observed (Fig. 1), an A at position 10a that is not paired and the absence of the conserved U12–A24 base pair, an essential identity element for *E. coli* GluRS [8]. Due to the presence of an A that is not paired, two alternative secondary structures are predicted for this tRNA (Fig. 1). While structure 1 contains four base pairs in the D stem, structure 2 has only three base pairs.

Sequencing of the mature tRNA by an RT-PCR based approach confirmed the presence of the A10a (data not shown). While this experiment confirmed the expression of the $tRNA_3^{Glu}$ Northern analysis showed a low level compared to other $tRNA^{Glu}$ (data not shown).

3.2. $tRNA_3^{Glu}$ is substrate of GluRS1 but not of GluRS2

To test the glutamylation of $tRNA_3^{Glu}$, we first analyzed the in vitro charging of the transcript tRNA with purified *A. ferrooxidans* GluRS1 or GluRS2. While in vitro transcribed $tRNA_3^{Glu}$ is aminoacylated by GluRS1 (Fig. 2) to the same extent as the tRNA expressed in vivo in *E. coli* (data not shown), GluRS2 is unable to charge this tRNA irrespective of how it was obtained. These results confirm that differential identity elements are recognized by the two GluRS enzymes [12]. Compared to other substrates of GluRS1 ($tRNA_1^{Glu}$ and $tRNA_2^{Glu}$) $tRNA_3^{Glu}$ is charged with lower efficiency (Fig. 2).

Due to the uncertain architecture of the D stem, we designed two mutants of $tRNA_3^{Glu}$: mutant 1 with a deletion of the A10a (predicted four base pair at the D stem), and mutant 2, with deletions of the A10a and C10–G25 (predicted three base pair at the D stem). The mutant tRNA gene transcripts were tested as substrates of both GluRSs. While mutant 1 is charged slightly more efficiently than wild type $tRNA_3^{Glu}$, mutant 2 is not charged by GluRS1 (Fig. 2), in agreement with previously reported specificity [12,23]. GluRS2 does not charge any of the mutant transcript tRNAs. Based on these results we concluded that four base pairs may form the D stem in $tRNA_3^{Glu}$.

3.3. $tRNA_3^{Glu}$ binds to elongation factor Tu

Glu-$tRNA_3^{Glu}$ and Glu-$tRNA_1^{Glu}$ were tested for binding to *A. ferrooxidans* EF-Tu. A pull-down binding assay was carried out using GST · EF-Tu immobilized on a glutathione–agarose. *A. ferrooxidans* EF-Tu binds each Glu-$tRNA^{Glu}$ to the same extent (Table 1). Binding of Glu-$tRNA^{Glu}$ is 12–15 fold higher than the binding of *A. ferrooxidans* Glu-$tRNA^{Gln}$ used as control. This result strongly suggests that Glu-$tRNA_3^{Glu}$ as well as Glu-$tRNA_1^{Glu}$ are substrates for protein biosynthesis.

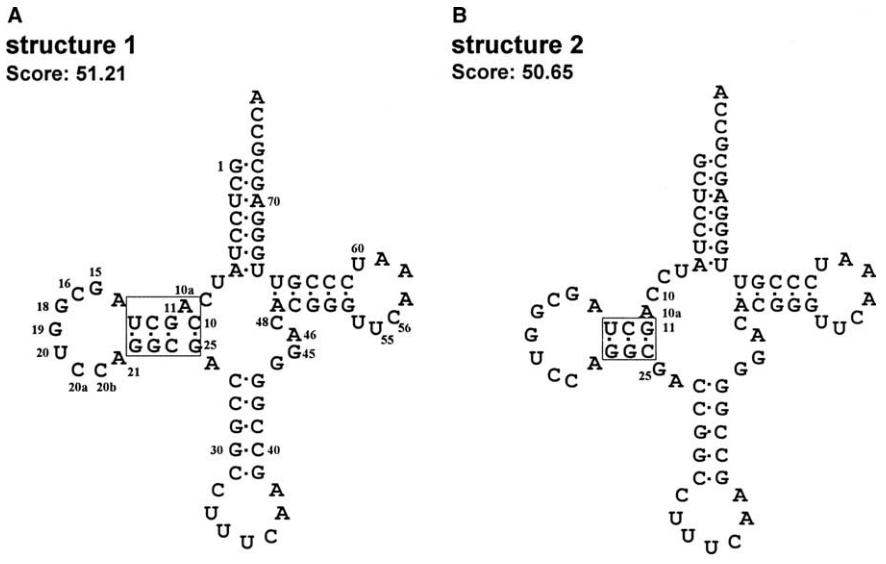

Fig. 1. Predicted cloverleaf structures of *A. ferrooxidans* $tRNA_3^{Glu}$. Structure 1 with four base pairs (A) and structure 2 with three base pairs (B) in the D stem (boxed), respectively. Score values for each structure obtained with tRNAScan program are shown. Numbering of nucleotides is in accordance to the crystal structure of yeast $tRNA^{Phe}$.

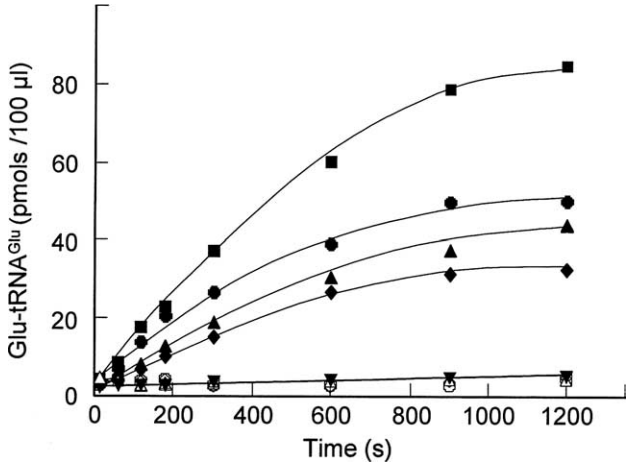

Fig. 2. Aminoacylation of *A. ferrooxidans* $tRNA^{Glu}$. $tRNA_1^{Glu}$ (■), $tRNA_2^{Glu}$ (●), $tRNA_3^{Glu}$ wt (◆), $tRNA_3^{Glu}$ mutant 1 (▲) and mutant 2 (▼) were acylated by GluRS1. Open symbols correspond to control without enzyme. Standard deviation for each determination was not higher than 10%.

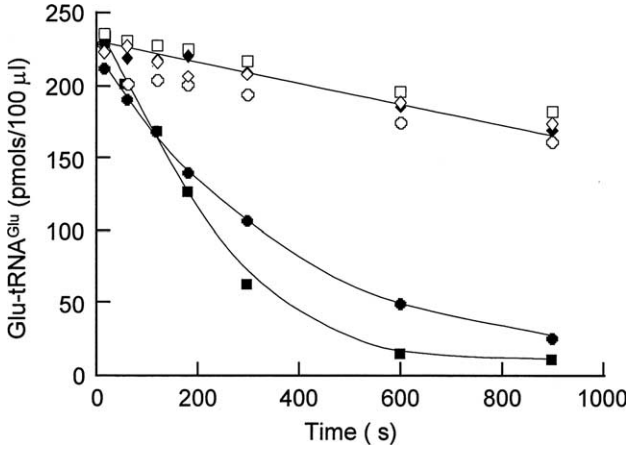

Fig. 3. GluTR activity. Glu-$tRNA_1^{Glu}$ (■), Glu-$tRNA_2^{Glu}$ (●) and Glu-$tRNA_3^{Glu}$ (◆) remaining after depletion by *A. ferrooxidans* GluTR. Open symbols correspond to control without enzyme. Standard deviation for each determination was not higher than 7%.

Table 1
Binding of Glu-tRNA to A. ferrooxidans EF-Tu

	Glu-tRNA bound (pmol)[a]
Glu-$tRNA_1^{Glu}$ (86)[b]	8.11 ± 1.79
Glu-$tRNA_3^{Glu}$ (74)[b]	9.68 ± 0.72
Glu-$tRNA^{Gln}$ (76)[b]	0.63 ± 0.20

[a]Picomoles of aa-tRNA eluted from EF-Tu. Numbers represent the average of two independent experiments.
[b]Concentration of aa-tRNA in each reaction mixture.

3.4. $tRNA_3^{Glu}$ is not substrate of GluTR

Glu-$tRNA_1^{Glu}$, Glu-$tRNA_2^{Glu}$ and Glu-$tRNA_3^{Glu}$ were tested for specificity of *A. ferrooxidans* GluTR by the depletion assay [21]. As shown in Fig. 3, Glu-$tRNA_1^{Glu}$ and Glu-$tRNA_2^{Glu}$, but not Glu-$tRNA_3^{Glu}$ are substrates of GluTR. Similar results were obtained using either transcripts or tRNAs overexpressed in vivo in *E. coli* (data not shown) indicating that chemical modifications are not responsible for the inability of Glu-$tRNA_3^{Glu}$ to serve as substrate of GluTR. Furthermore, GluTR activity with Glu-$tRNA_1^{Glu}$ or Glu-$tRNA_2^{Glu}$ is not affected when a 10-fold excess of Glu-$tRNA_3^{Glu}$ is added to the reaction mixture (data not shown) suggesting that GluTR does not bind Glu-$tRNA_3^{Glu}$.

Although all the identity elements of *E. coli* $tRNA^{Glu}$ for recognition by GluTR [9] are present in $tRNA_3^{Glu}$, some sequence differences with other tRNAs substrate of *A. ferrooxidans* GluTR exist ($tRNA_1^{Glu}$, $tRNA_2^{Glu}$ and *E. coli* $tRNA^{Glu}$). In addition to mutant 1 (Section 3.2) four mutants (that were combinations of the following changes: C10–G25 → G10–C25, G11–C24 → U11–A24, G44G45 → U44A45, A57 → G57) were designed to bring $tRNA_3^{Glu}$ toward the consensus of GluTR substrates. While all mutant tRNAs were efficiently

charged by GluRS1, none was deacylated by GluTR (data not shown). These results might indicate that a yet unidentified distortion of the tertiary structure of Glu-tRNA$_3^{Glu}$ is responsible of the effect on the recognition by GluTR.

Thus, due to the fact that Glu-tRNA$_3^{Glu}$ is not substrate of GluTR but it efficiently binds to EF-Tu factor, we conclude that the formation of Glu-tRNA$_3^{Glu}$ might ensure the availability of Glu-tRNA for protein translation under conditions of high demand of heme.

4. Discussion

Most organisms contain only one tRNA^{Glu} gene, thus GluTR, considered the first enzyme committed to tetrapyrrole biosynthesis, must compete with EF-Tu for the Glu-tRNA^{Glu}. In model organisms as *E. coli* only a small amount of Glu-tRNA is shunted to tetrapyrrole biosynthesis. Thus, no detrimental effect on protein biosynthesis is expected upon heme biosynthesis. Conversely in organisms like *A. ferrooxidans,* that use high redox potential substrates for respiration, a vast amount of cytochromes (and hence heme) are formed [24,10] without any detrimental effect on protein synthesis. The existence in *A. ferrooxidans* of at least three different tRNA^{Glu} species encoded by independent genes, suggested that each tRNA might have different roles. Our data demonstrate that one such tRNA^{Glu}(tRNA$_3^{Glu}$) is properly aminoacylated by one of duplicated GluRSs existing in this organism, but is not substrate of GluTR. Thus, this tRNA might participate in protein but not in tetrapyrrole biosynthesis. This prediction is supported by the efficient binding of Glu-tRNA$_3^{Glu}$ to the EF-Tu. The inability of Glu-tRNA$_3^{Glu}$ to function as substrate of GluTR appears to be not only the result of a mutation that alters the tRNA core structure; none of the mutations designed to restore the core structure resulted in Glu-tRNA suitable for GluTR activity, although their aminoacylation capacity was maintained. We believe that a global yet unidentified structural distortion might account for this effect.

Thus, aminoacylation of tRNA$_3^{Glu}$, an independently encoded tRNA rather than the result of a mutant tRNA gene [25], might ensure the provision of Glu-tRNA^{Glu} for protein biosynthesis upon conditions of high heme demand by an uncoupling of protein and tetrapyrrole biosynthesis. Since the tRNA under scrutiny is expressed at low levels, several questions arise. Among them the following are relevant: Is there any cell condition where this tRNA is expressed at higher levels? If there is any, what are the signals for such a regulation of the tRNA expression? Are there physical or functional interactions that channel the Glu-tRNA products of the two GluRSs to a certain process? Are there any other roles for this tRNA? Future research will address these open questions.

Acknowledgements: We thank to J.C. Salazar and L. Randau for assistance and generous gift of plasmids and reagents. This work was supported by grants by Fondecyt, Chile (1020087) (to O.O.) and from the National Institute of General Medical Sciences, and Department of Energy (to D.S.).

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