

Article

Effect of Hydrogen Peroxide on the Biosynthesis of Heme and Proteins: Potential Implications for the Partitioning of Glu-tRNA^{Glu} between These Pathways

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Abstract: Glutamyl-tRNA (Glu-tRNA^{Glu}) is the common substrate for both protein translation and heme biosynthesis via the C₅ pathway. Under normal conditions, an adequate supply of this aminoacyl-tRNA is available to both pathways. However, under certain circumstances, Glu-tRNA^{Glu} can become scarce, resulting in competition between the two pathways for this aminoacyl-tRNA. In *Acidithiobacillus ferrooxidans*, glutamyl-tRNA synthetase 1 (GluRS1) is the main enzyme that synthesizes Glu-tRNA^{Glu}. Previous studies have shown that GluRS1 is inactivated *in vitro* by hydrogen peroxide (H₂O₂). This raises the question as to whether H₂O₂ negatively affects *in vivo* GluRS1 activity in *A. ferrooxidans* and whether Glu-tRNA^{Glu} distribution between the heme and protein biosynthesis processes may be affected by these conditions. To address this issue, we measured GluRS1 activity. We determined that GluRS1 is inactivated when cells are exposed to H₂O₂, with a concomitant reduction in intracellular heme level. The effects

of H₂O₂ on the activity of purified glutamyl-tRNA reductase (GluTR), the key enzyme for heme biosynthesis, and on the elongation factor Tu (EF-Tu) were also measured. While exposing purified GluTR, the first enzyme of heme biosynthesis, to H₂O₂ resulted in its inactivation, the binding of glutamyl-tRNA to EF-Tu was not affected. Taken together, these data suggest that in *A. ferrooxidans*, the flow of glutamyl-tRNA is diverted from heme biosynthesis towards protein synthesis under oxidative stress conditions.

Keywords: heme; oxidative stress; acidophilic; aminoacyl-tRNA

1. Introduction

Heme is a fundamental molecule for living organisms, as the cofactor for several proteins and enzymes involved in cellular processes, such as transport of gases, redox reactions and electron transport [1,2]. Despite its significant roles, heme synthesis must be carefully balanced due to the potentially toxic effects of both heme itself and its intermediates [3–6]. Heme synthesis occurs by a universally conserved metabolic pathway for synthesizing tetrapyrroles, which starts with δ -aminolevulinic acid (ALA) [1,2]. There are two ways to synthesize ALA. While animals, fungi and α -proteobacteria use the Shemin pathway, plants, archaea and most bacteria use the C₅ pathway [2]. In the Shemin pathway, ALA is formed from the condensation of glycine and succinyl-CoA, a reaction that is catalyzed by ALA synthase (ALAS). In the C₅ pathway, ALA is synthesized from Glu-tRNA^{Glu} in two steps. First, the glutamate moiety of Glu-tRNA^{Glu} is reduced to glutamate semialdehyde (GSA) by glutamyl-tRNA reductase (GluTR), and then GSA is converted to ALA by the glutamate semialdehyde 1-2 aminomutase (GSAM) [1,2]. In organisms that use the C₅ pathway, Glu-tRNA^{Glu} is a common substrate for heme and protein synthesis and must be partitioned between these two processes [7,8]. This distribution is likely determined at least partially by the demand for Glu-tRNA^{Glu} from each pathway, which in turn depends on the physiology of each particular organism. In chemolithoautotrophic bacteria like *Acidithiobacillus ferrooxidans* that use the C₅ pathway to synthesize tetrapyrroles, high demand for Glu-tRNA^{Glu} for heme biosynthesis is expected, due to the high cytochrome content required for respiration using poor electron donors, such as ferrous ions [9,10]. This bacterium has a complex system of glutamyl-tRNA formation composed of two non-discriminating glutamyl-tRNA synthetases (GluRS1 and GluRS2) and up to four tRNA^{Glu} isoacceptors, with GluRS1 serving as the main enzyme for Glu-tRNA^{Glu} formation [9,11,12]. Three out of four glutamyl-tRNAs can act as donors for both heme and protein synthesis, while the fourth is not a substrate of GluTR and likely acts exclusively in protein synthesis [12]. The above underscores the importance of maintaining a balanced Glu-tRNA^{Glu} supply to each of these metabolic pathways in this organism. Under conditions that reduce the supply of Glu-tRNA^{Glu}, both heme and protein synthesis could be compromised. Thus, increased competition between heme and protein biosynthesis pathways for this substrate might change the distribution of Glu-tRNA^{Glu} toward one process or the other. Since it is known that *A. ferrooxidans* GluRS1 is inactivated *in vitro* by H₂O₂ [13], we wondered whether inactivating this enzyme might also occur *in vivo*, potentially lowering the pool of Glu-tRNA^{Glu} in the cell and affecting the partitioning of Glu-tRNA^{Glu} towards heme vs. protein synthesis. To investigate this question, we

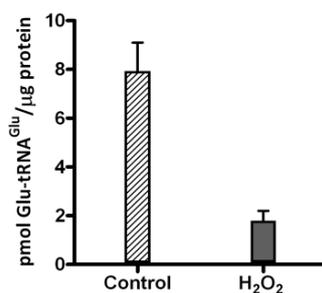
determined the effect of H₂O₂ on GluRS1 activity and heme content in *A. ferrooxidans*. We found that treating *A. ferrooxidans* with H₂O₂ effectively decreased the intracellular activity of GluRS1 and correlated with reduced intracellular heme levels. To provide insight into the fate of Glu-tRNA^{Glu} under this condition, we determined the *in vitro* effect of H₂O₂ on the deacylation of Glu-tRNA^{Glu} by GluTR and the binding of Glu-tRNA^{Glu} to elongation factor Tu (EF-Tu). We observed that upon treatment with H₂O₂, the activity of purified GluTR, as well as its activation by GSAM, was reduced, while the binding of Glu-tRNA^{Glu} to EF-Tu was not affected. Whether these effects of H₂O₂ on GluTR and EF-Tu occur *in vivo* is yet to be determined. We speculate that oxidative stress may reduce intracellular Glu-tRNA^{Glu} concentration, potentially further reducing the partition of this aminoacyl-tRNA towards heme synthesis. Conversely, distribution of the substrate towards protein synthesis might be less affected.

2. Results and Discussion

2.1. Hydrogen Peroxide Inactivates Glutamyl-tRNA Synthetase 1 (GluRS1) and Reduces Heme Levels in *A. ferrooxidans*

Given that GluRS1 is inactivated by H₂O₂ *in vitro* [13], we sought to determine whether GluRS1 activity is decreased *in vivo* in *A. ferrooxidans* exposed to H₂O₂. Cellular extracts from *A. ferrooxidans* treated with 1 mM H₂O₂ were obtained, and the specific activity of GluRS1 was evaluated in these extracts by the aminoacylation of tRNA₂^{Glu}, a specific substrate of GluRS1 [9,11]. Glu-tRNA₂^{Glu} formation was decreased by around 80% in this extract relative to the control (Figure 1). Thus, we can conclude that H₂O₂ also inactivates GluRS1 *in vivo*. Along with inactivating GluRS1, treating *A. ferrooxidans* with H₂O₂ also affected heme levels. After treating the cells with H₂O₂ for 2.5 h, intracellular heme concentration fell from 17.2 ± 0.75 pmol/μg of protein in control cells to 12.9 ± 0.23 pmol/μg. Inactivating GluRS1 by H₂O₂ exposure, then may reduce intracellular availability of Glu-tRNA^{Glu}. Under such conditions, competition between the heme and protein biosynthesis pathways for Glu-tRNA^{Glu} may increase. As a first step to test this hypothesis, we evaluated the effect of H₂O₂ on the deacylation of Glu-tRNA^{Glu} by GluTR and its binding to EF-Tu (see below).

Figure 1. Glutamyl-tRNA synthetase 1 (GluRS1) activity in *A. ferrooxidans* extracts. Specific activity of GluRS1 in extracts from *A. ferrooxidans* treated with 1 mM H₂O₂ for 30 min was measured. Control extract was prepared from cells not treated with H₂O₂. The mean ± SD values for pmol of Glu-tRNA₂^{Glu}/μg protein formed after 15 min in three different extracts for each condition are shown.



2.2. Glutamyl-tRNA Reductase (GluTR) from *A. ferrooxidans* Is Inactivated by Hydrogen Peroxide

GluTR is the first enzyme committed to the biosynthesis of heme and is a key regulatory enzyme of the C₅ pathway [14–16]. Therefore, in order to determine whether Glu-tRNA^{Glu} is diverted from heme biosynthesis under experimental conditions, we determined the effect of H₂O₂ on the activity of the recombinant purified enzyme. The deacylation of Glu-tRNA₂^{Glu} catalyzed by GluTR was used to measure the enzymatic activity. We found that the presence of H₂O₂ decreased GluTR activity (Figure 2). Additionally, we observed that higher levels of heme bound to *A. ferrooxidans* GluTR [17] enhanced inactivation by H₂O₂. Enzyme preparations with one molecule of heme bound per four GluTR subunits (heme/protein ratio of 1/4) had an increased inactivation rate compared to enzymes with one heme per twelve GluTR subunits (heme/protein ratio of 1/12) (Table 1).

Figure 2. Inactivation of glutamyl-tRNA reductase (GluTR) by H₂O₂. Recombinant purified GluTR from *A. ferrooxidans* was incubated with H₂O₂ for 20 min at 37 °C. After treatment, activity was measured as the depletion of Glu-tRNA₂^{Glu} by GluTR (1 μM) that was untreated (●) or treated with 250 μM (▲) or 500 μM H₂O₂ (Δ). Black squares (■) represent the control without enzyme. Each curve represents the mean of three independent determinations. For each point, standard deviation was no higher than 13%.

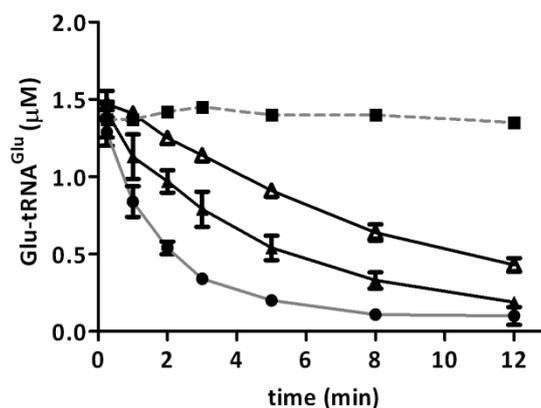


Table 1. Inactivation rates (k_{obs}) of GluTR by H₂O₂. Numbers in parentheses represent the relative standard deviation of three experimental replications. k_{obs} is the first-order inactivation rate obtained from the slope of $\ln N/N_0$ against time. For detailed procedures, see Materials and Methods (GluTR Activity).

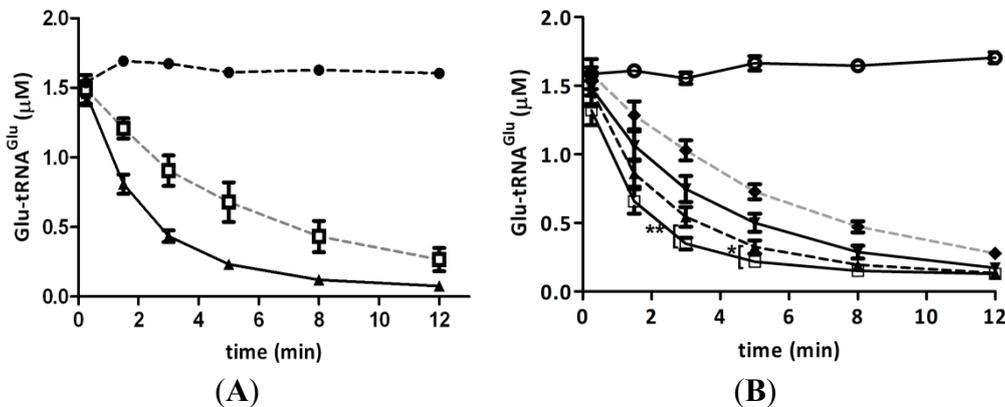
Type of GluTR (H ₂ O ₂)	GluTR (1/4) 250 μM H ₂ O ₂	GluTR (1/4) 500 μM H ₂ O ₂	GluTR (1/12) 500 μM H ₂ O ₂
k_{obs} (s ⁻¹)	$3.18 \times 10^{-4} \pm (4.43 \times 10^{-5})$	$6.96 \times 10^{-4} \pm (4.49 \times 10^{-5})$	$4.82 \times 10^{-4} \pm (2.29 \times 10^{-5})$

2.3. Hydrogen Peroxide Decreases the Stimulation of GluTR by Glutamate Semialdehyde 1-2 Aminomutase (GSAM)

In *E. coli* and *Chlamydomonas reinhardtii*, GluTR activity is stimulated upon formation of a complex with GSAM [18,19]. As the amount of Glu-tRNA^{Glu} devoted to heme biosynthesis depends on GluTR activity, we evaluated whether recombinant GluTR activity is stimulated by recombinant

A. ferrooxidans GSAM. We found that GSAM effectively stimulated GluTR activity (Figure 3A). This effect was observed only when GluTR contained a heme/protein ratio of 1/12. To further investigate whether H₂O₂ affected the stimulation of GluTR by GSAM, the deacylation of Glu-tRNA^{Glu} was assayed after treating these enzymes with H₂O₂. As shown in Figure 3B, the stimulation of GluTR by GSAM was reduced when GSAM was treated with H₂O₂. These data, in turn, indicated that both GluTR activity and the stimulation of this enzyme by GSAM were reduced by treatment with H₂O₂. If these effects take place *in vivo*, a reduced partitioning of Glu-tRNA^{Glu} to heme biosynthesis might occur after treating *A. ferrooxidans* with H₂O₂. These results are consistent with the reduced heme levels observed in cells treated with H₂O₂ (see above).

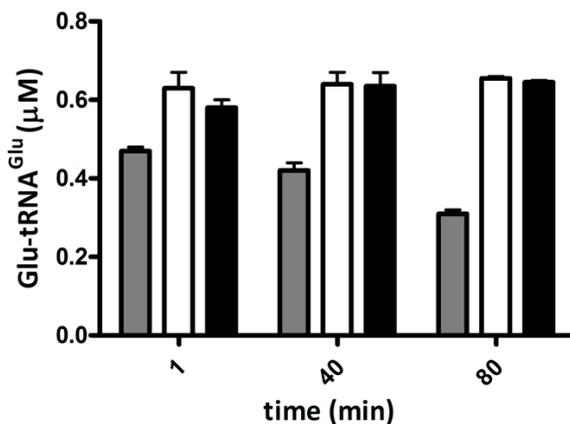
Figure 3. Stimulation of GluTR by glutamate semialdehyde 1-2 aminomutase (GSAM). (A) The effect of GSAM on GluTR activity was analyzed by determining the deacylation of Glu-tRNA^{Glu} catalyzed by GluTR (1 μM heme/protein ratio of 1/12) alone (□) or in the presence of GSAM (▲). Black circles (●) correspond to deacylation of Glu-tRNA^{Glu} without enzyme; (B) The effect of H₂O₂ on GSAM stimulation of GluTR activity was evaluated by the deacylation of Glu-tRNA^{Glu} catalyzed by GluTR (1 μM, heme/protein ratio of 1/12) in the presence of GSAM when GluTR (▼), GSAM (▲) or both enzymes (◆) were treated with 300 μM H₂O₂. Control (□) represents enzyme not treated with H₂O₂. Open circles (○) correspond to deacylation of Glu-tRNA^{Glu} without enzymes. Asterisks represent statistically significant differences (** *p* = 0.008; * *p* = 0.035).



2.4. Elongation Factor Tu (EF-Tu) Is not Affected by Treatment with H₂O₂

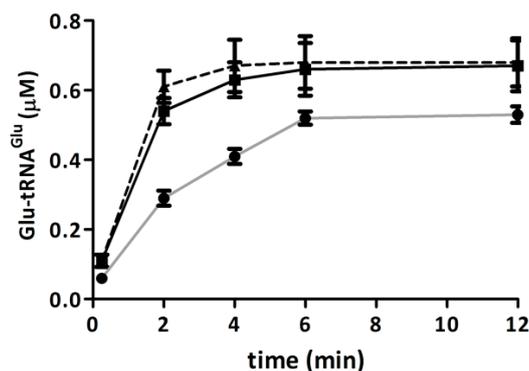
The amount of Glu-tRNA^{Glu} dedicated to protein synthesis depends in part on its binding to EF-Tu. As a measure of Glu-tRNA^{Glu} binding to EF-Tu, we assessed the protection of Glu-tRNA^{Glu} by EF-Tu against spontaneous deacylation. Purified recombinant EF-Tu from *A. ferrooxidans* was incubated with H₂O₂, and then the ability of this protein to protect Glu-tRNA^{Glu} from spontaneous deacylation was tested. EF-Tu treated with H₂O₂ was still able to protect Glu-tRNA^{Glu} from deacylation (Figure 4), implying that the ability of EF-Tu to bind Glu-tRNA^{Glu} was not altered by H₂O₂.

Figure 4. Protection of Glu-tRNA^{Glu} by elongation factor Tu (EF-Tu) against deacylation. Remaining Glu-tRNA^{Glu} over time was determined in the presence of EF-Tu, treated with 500 μ M H₂O₂ (black bars) or untreated (open bars) at 37 °C. The spontaneous deacylation of Glu-tRNA^{Glu} without EF-Tu was used as a control (gray bars).



Since class I aminoacyl-tRNA synthetases are stimulated by EF-Tu [20], and this stimulation depends on the binding of the aminoacyl-tRNA to EF-Tu, we measured the effect of H₂O₂ on the activation of purified recombinant GluRS1 by purified recombinant EF-Tu. We observed that EF-Tu exposed to H₂O₂ retained its ability to stimulate GluRS1 activity (Figure 5). Taken together, the data suggest that treating recombinant purified EF-Tu with H₂O₂ does not affect the binding of Glu-tRNA^{Glu}.

Figure 5. Activity of GluRS1 in presence of EF-Tu. The aminoacylation of tRNA^{Glu} catalyzed by GluRS1 was determined in the absence (●) or presence of EF-Tu, treated with 500 μ M H₂O₂ (▲) or untreated (■).

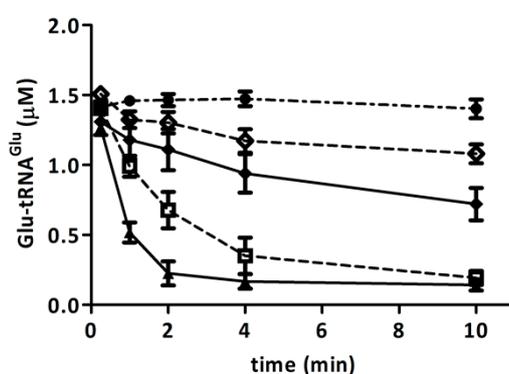


2.5. Competition of GluTR and EF-Tu for Glu-tRNA^{Glu} Is Affected by H₂O₂

The observation that, after treatment with H₂O₂, recombinant GluTR was inactivated while recombinant EF-Tu maintained its ability to bind Glu-tRNA^{Glu}, led us to test to whether EF-Tu would compete with GluTR for Glu-tRNA^{Glu}. We evaluated the protection of Glu-tRNA^{Glu} by EF-Tu against the deacylation catalyzed by GluTR. We found that EF-Tu effectively protected Glu-tRNA^{Glu} from deacylation by GluTR, indicating that GluTR and EF-Tu compete for Glu-tRNA^{Glu} (Figure 6). Furthermore, when GluTR and EF-Tu were both treated with H₂O₂, there was increased protection

against the deacylation of Glu-tRNA^{Glu} (Figure 6). In the event that this competition takes place *in vivo*, it is possible that under oxidative stress, Glu-tRNA^{Glu} would be diverted from the heme biosynthesis pathway.

Figure 6. Competition between GluTR and EF-Tu for Glu-tRNA^{Glu}. The deacylation of Glu-tRNA₂^{Glu} catalyzed by GluTR (0.5 μM) in presence of EF-Tu is shown. Deacylation was monitored when GluTR was untreated (▲,◆) or previously treated (□,◇) with 300 μM H₂O₂ and in the presence of EF-Tu, treated (◇) or untreated (◆) with 300 μM H₂O₂. The deacylation of Glu-tRNA₂^{Glu} in the absence of GluTR or EF-Tu was used as a control (●). Each curve is the mean of three experimental replications. For each point, the standard deviation was no higher than 15%.



2.6. Discussion

Glu-tRNA^{Glu} is a shared substrate for the biosynthetic pathways of proteins and porphyrins. Therefore, these pathways likely compete for Glu-tRNA^{Glu}. Indeed, it has been described that partial inhibition of protein synthesis increases heme production [7]. This competition might be more pronounced under conditions that affect the availability of Glu-tRNA^{Glu}. *A. ferrooxidans* is a chemolithotrophic bacterium with an elevated requirement for Glu-tRNA^{Glu} for the synthesis of heme used in respiration processes. We previously demonstrated that GluRS1, the main enzyme that generates Glu-tRNA^{Glu} for heme and protein synthesis in *A. ferrooxidans*, is inactivated by H₂O₂. Here we have shown that endogenous GluRS1 is also inactivated upon treatment of the cells with H₂O₂. This inactivation of GluRS1 in cells exposed to H₂O₂ might imply a reduction in intracellular Glu-tRNA^{Glu} levels. A concomitant reduction in heme levels in *A. ferrooxidans* after treatment with H₂O₂ was also observed. In *E. coli*, reduced GluRS activity was also correlated with decreased Glu-tRNA^{Glu} levels [21]. Reduced Glu-tRNA^{Glu} availability could increase competition for this substrate and thereby affect its partition to heme and protein biosynthetic pathways. Thus, we hypothesize that oxidative stress might enhance competition between these biosynthetic pathways for use of Glu-tRNA^{Glu}. Our data using recombinant purified proteins from *A. ferrooxidans* revealed that upon treatment with H₂O₂, both GluTR activity and GluTR stimulation by GSAM are reduced. Furthermore, we observed that H₂O₂ had no effect on the binding of Glu-tRNA^{Glu} to EF-Tu. When competing for the substrate, treatment of these molecules with H₂O₂ favored the binding of Glu-tRNA^{Glu} to EF-Tu over GluTR. Although it remains to be shown empirically, these data lead us to speculate that under oxidative stress, the partitioning of Glu-tRNA^{Glu} to protein translation might

be favored over heme biosynthesis. Although increased heme turnover cannot be ruled out, this conjecture correlates well with the lower heme levels in cells treated with H₂O₂. Since heme and its precursors generate ROS [3–6], reduced partitioning of Glu-tRNA^{Glu} to heme biosynthesis in *A. ferrooxidans* could favor cell survival when exposed to oxidative stress. It has been described that in *Cucumis sativus*, the ROS producer methyl viologen decreases ALA synthesis, likely by inactivating GluTR and inhibiting GluTR–GSAM complex formation [22]. Furthermore, other mechanisms to avoid the accumulation of toxic heme intermediates exist. In *Salmonella enteric* and *Rhodobacter sphaeroides*, H₂O₂ affects the transcription of genes for heme biosynthesis. In *S. enteric*, H₂O₂ decreases the transcription of *hemA*, which encodes GluTR, and induces the expression of *hemH*, which encodes ferrochelatase, the last enzyme in the heme biosynthetic pathway [23]. In *R. sphaeroides*, *hemA* is down-regulated when this bacterium is treated with H₂O₂ [24]. In *B. subtilis*, a mutation in *per* increases expression of the operon *hemAXCDBL*, which encodes the enzymes for heme biosynthesis. Secretion of overproduced porphyrins then takes place, preventing toxicity [25]. Thus, different strategies seem to have been developed in nature to overcome the potential negative effects of the accumulation of heme and its intermediates, particularly under oxidative stress conditions.

3. Experimental Section

3.1. Bacterial Strains and Culture Conditions

A. ferrooxidans ATCC 23270 was grown aerobically at 30 °C in a modified 9K medium (0.40 g/L MgSO₄·7H₂O, 0.10 g/L (NH₄)₂SO₄, 0.04 g/L K₂HPO₄·3H₂O and 33.3 g/L FeSO₄). For production and purification of recombinant proteins, *E. coli* BL21 [DE3] or JM109 were grown in Luria–Bertani medium at 37 °C. When necessary, 100 µg/mL ampicillin were added to LB medium.

3.2. Overproduction and Purification of Proteins

All recombinant proteins from *A. ferrooxidans* were overexpressed in *E. coli* BL21 [DE3]. Recombinant GluRS and EF-Tu were expressed as GST-fusion proteins and purified by affinity chromatography using glutathione agarose as described [9,12,13,17]. After purification, the Glutathione-S-Transferase (GST) tag was removed by digestion with thrombin according to the manufacturer's instructions. Purification of His₆-tagged GluTR and GSAM was carried out with a Ni²⁺ affinity resin column. To obtain recombinant GluTR with reduced heme content, the protein was expressed in *E. coli* BL21 [DE3] in the presence of 100 µM orthophenanthroline before inducing expression with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) [9,17]. Purity of proteins was assessed by polyacrylamide-SDS gel electrophoresis and Coomassie blue staining. One major protein band was observed in all purified proteins except in GSAM, in which one major protein (>80%) of the expected molecular mass and two minor proteins were detected.

3.3. Preparation and Purification of tRNA Transcripts

A. ferrooxidans tRNA₂^{Glu} (UUC) was prepared by *in vitro* transcription [26]. The DNA template was prepared by PCR amplification of the tRNA₂^{Glu} gene cloned in pUC18. The *in vitro* transcription reaction mixture contained 40 mM Tris–HCl pH 8.0, 22 mM MgCl₂, 2 mM spermidine, 5 mM

dithiothreitol (DTT), 0.05 mg/mL bovine serum albumin (BSA), 4 mM each nucleotide triphosphate (NTP), 20 mM guanosine monophosphate (GMP), 1 U pyrophosphatase, 25 ng/ μ L tRNA^{Glu} and 0.12 ng/ μ L T7 RNA polymerase. The reaction was incubated for 5 h at 42 °C. Afterwards, the tRNA was purified by anionic exchange using Qiagen-tip100 columns following the recommendations of the manufacturer.

3.4. Preparation of *A. ferrooxidans* Cellular Extracts

A. ferrooxidans cells were collected by centrifugation, and the pellet was suspended in 10 mM H₂SO₄ and treated with 1 mM H₂O₂ for 30 min at 30 °C. After exposure to H₂O₂, the cells were washed twice with 10 mM H₂SO₄ and suspended in 60 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 8, 300 mM NaCl. The cell suspension was sonicated and the cellular debris removed by centrifugation at 15,000 \times g for 30 min. Next, the supernatant was ultracentrifuged at 150,000 \times g for 90 min to obtain cellular extracts. Proteins were measured using the Bradford procedure and the cellular extracts stored in aliquots at -80 °C until use.

3.5. GluRS Activity

The activity of endogenous GluRS1 from *A. ferrooxidans* was measured in cellular extracts with buffer containing 5 mM ATP, 100 μ M [¹⁴C] Glu, 100 mM HEPES KOH pH 7.2, 30 mM KCl, 12 mM MgCl₂, 3.7 μ g cellular extract and 8.4 μ M tRNA^{Glu}. The activity of recombinant purified GluRS1 from *A. ferrooxidans* was measured at 37 °C in 100 mM HEPES KOH pH 7.2, 30 mM KCl, 12 mM MgCl₂, 5 mM ATP, 50 μ M [¹⁴C] Glu and 0.1 μ M GluRS1 [11,13]. The reaction was started by adding 2.6 μ M tRNA^{Glu}. Aliquots were removed at different times, [¹⁴C] Glu-tRNA^{Glu} was precipitated with 15% trichloroacetic acid (TCA), and radioactivity was measured in a scintillation counter. When GluRS1 activity in the presence of EF-Tu was measured, 5 μ M EF-Tu treated with or without H₂O₂ was included.

3.6. GluTR Activity

The activity of purified GluTR was determined by measuring the deacylation of Glu-tRNA^{Glu}. Because of the instability of the reaction product glutamate semialdehyde, it is described as the method of choice to assess the activity of this enzyme [9,15,17,22]. Aminoacylated [¹⁴C] Glu-tRNA^{Glu} was prepared with GluRS1 using 40 μ M [¹⁴C] Glu and 10 μ M tRNA^{Glu}. After preparation, [¹⁴C] Glu-tRNA^{Glu} was precipitated and stored at -80 °C. GluTR activity was measured at 37 °C in 30 mM HEPES-KOH pH 7.2, 4 mM KCl, 1.5 mM MgCl₂, 0.1 μ g/ μ L BSA, 10% glycerol, 2 mM NADPH, 0.5–1.0 μ M GluTR and 1–2 μ M [¹⁴C] Glu-tRNA^{Glu}. The reaction was started by adding [¹⁴C] Glu-tRNA^{Glu}, and at different times aliquots were removed and the remaining [¹⁴C] Glu-tRNA^{Glu}/mg protein measured after precipitation with 15% TCA. To measure the inactivation rate of GluTR by H₂O₂, the reaction was started by adding H₂O₂, and at different times, aliquots were taken to determine GluTR activity. The remaining GluTR activity was determined by comparing the enzyme activity of the treated GluTR with the activity of the enzyme under the same conditions without the addition of H₂O₂. The remaining activity was plotted as $\ln N/N_0$ against time, where N_0 and N are activity at time

zero and at a given time, respectively. The slope of the lines obtained represents the first-order inactivation rate (k_{obs}). Competition assays between GluTR and EF-Tu for Glu-tRNA^{Glu} were carried out by measuring GluTR activity in the presence of 10 μM EF-Tu. The effect of GSAM (9 μM) on GluTR activity was measured by adding 0.1 mM pyridoxal phosphate.

3.7. Binding of Glutamyl-tRNA^{Glu} to EF-Tu

To evaluate the binding of EF-Tu to Glu-tRNA, we measured the spontaneous deacylation of [¹⁴C] Glu-tRNA₂^{Glu} in the presence of EF-Tu as described [27,28]. To activate EF-Tu, the protein was incubated at 37 °C for 30 min in a reaction mixture containing 500 mM HEPES–KOH pH 7.2, 200 mM MgCl₂, 0.8 M NH₄Cl, 4.3 mM GTP, 3.2 μM phosphoenolpyruvate and 0.15 μM pyruvate kinase. The mixture for measuring the deacylation of Glu-tRNA₂^{Glu} in the presence of EF-Tu contained 30 mM HEPES–KOH pH 7.2, 4 mM KCl, 1.5 mM MgCl₂, 0.1 $\mu\text{g}/\mu\text{L}$ BSA, 1 μM Glu-tRNA₂^{Glu} and 5 μM EF-Tu. The assay was started by adding EF-Tu and was performed at 37 °C.

3.8. Protein Oxidation

Treatment of GluTR, EF-Tu and GSAM with H₂O₂ was performed at 37 °C for 20 min in 100 mM HEPES–KOH pH 7.2, 30 mM KCl, 12 mM MgCl₂ and 250–500 μM H₂O₂. After treatment, H₂O₂ was removed by incubation with 0.1 nM catalase for 5 min at 37 °C.

3.9. Heme Measurement

Heme content was measured in the 12,000 \times g supernatant of formic acid extracts from *A. ferrooxidans* as absorbance at 398 nm [29] in an Epoch spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA).

4. Conclusions

The data presented here show that H₂O₂ reduces heme levels and inactivates GluRS in *A. ferrooxidans* cells. Furthermore, our results concerning the effect of H₂O₂ on the enzymatic activity of GluTR, the stimulation exerted on GluTR by GSAM and the binding of Glu-tRNA^{Glu} to EF-Tu allowed us to establish a model in which a limited supply of Glu-tRNA^{Glu} generated by oxidative stress this aminoacyl-tRNA changes its fate, favoring the channeling of Glu-tRNA^{Glu} to the biosynthesis of proteins instead of the biosynthesis of heme. Even though additional experiments *in vivo* are required to probe these statements and we cannot rule out alternative explanations, this could represent a way to avoid the accumulation of heme and its toxic intermediaries, which is more relevant under oxidative stress, when the cellular detoxifying system of oxidizing species is being depleted. In this context, beside the further investigation to establish how *in vivo*, the oxidative stress affects the delivery of Glu-tRNA^{Glu} towards the protein and heme syntheses would be interesting analyzed if the partition of this substrate to the tetrapyrroles biosynthesis is also affected in other organism with high demand of heme under oxidative stress. *Leptospirillum ferriphilum* is an adequate model since it to be characterized by their high heme content [9,10] and share with *A. ferrooxidans* an ecological niche with a high heavy metal content, which generates ROS [30–32]. However, both

bacteria have different strategies to respond to oxidative stress [33]. While in *A. ferrooxidans* a reduction in the production of ROS seems to be the major response, in *L. ferriphilum* the repairing of damaged molecules takes place [33].

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Author Contributions

Carolina Farah performed most of the experimental work and contributed to discussion of the results. Gloria Levicán provided her expertise in acidophilic microorganisms and to interpretation of the results. Michael Ibba provided his expertise in the enzymatic analysis of aminoacyl-tRNA-related enzymes; contributed to carrying out several of the experiments; and contributed to discussion of the results. Omar Orellana contributed to framing the general problem; designing and providing resources for the experiments; and carrying out the project.

Conflicts of Interest

The authors declare no conflict of interest.

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