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Cellular levels of heme affect the activity of dimeric glutamyl-tRNA reductase

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ABSTRACT

Glutamyl-tRNA reductase (GluTR) is the first enzyme committed to tetrapyrrole biosynthesis by the C_5-pathway. This enzyme transforms glutamyl-tRNA into glutamate-1-semi-aldehyde, which is then transformed into 5-amino levulinic acid by the glutamate-1-semi-aldehyde 2,1-aminomutase. Binding of heme to GluTR seems to be relevant to regulate the enzyme function. Recombinant GluTR from *Acidithiobacillus ferrooxidans* an acidophilic bacterium that participates in bioleaching of minerals was expressed in *Escherichia coli* and purified as a soluble protein containing type *b* heme. Upon control of the cellular content of heme in *E. coli*, GluTR with different levels of bound heme was obtained. An inverse correlation between the activity of the enzyme and the level of bound heme to GluTR suggested a control of the enzyme activity by heme. Heme bound preferentially to dimeric GluTR. An intact dimerization domain was essential for the enzyme to be fully active. We propose that the cellular levels of heme might regulate the activity of GluTR and ultimately its own biosynthesis.

1. Introduction

Heme is an essential molecule for most living organisms [1,2]. Heme is cofactor for gas transport [3], electron transport chains [4] and peroxide reduction [5]. Roles of heme as regulatory component of gene expression systems have been proposed [6–8]. Other important regulatory function of heme concerns to its own biosynthesis [9,10].

All tetrapyrroles, including heme, are biosynthesized from the universal precursor 5-aminolevulinic acid (ALA). There are two alternative routes in nature leading to ALA formation [1]. The Shemin pathway, present in non-photosynthetic eukaryotes and α-proteobacteria, requires one single step catalyzed by ALA synthase to form ALA from the condensation of succinyl-CoA and glycine [11]. In mammals, ALA synthase stability and enzyme activity are subjected to heme regulation [10]. In the C_5-pathway, found in most bacteria, archaea and plants, ALA is synthesized in a two-step reaction from glutamyl-tRNA^{Glu} (Glu-tRNA^{Glu}) [12,13]. In the first step, the tRNA-bound glutamate is reduced to glutamate-1-semi-aldehyde (GSA) in a NADPH-dependent reaction catalyzed by glutamyl-tRNA reductase (GluTR) [14,15]. In the second step, glutamate-1-semi-aldehyde 2,1-aminomutase (GSA-AT) mediates a pyridoxal-5′-phosphate-dependent transamination in GSA to yield ALA [15].

Stability and enzymatic activity of GluTR have been reported to be regulated by heme [9,16,17]. Recombinant GluTR from *Chlorobium vibrioforme* [18] and *Mycobacterium tuberculosis* [19] have been shown to bind heme, as well as wild-type GluTR but not C170A mutant, from *Salmonella enterica* serovar Typhimurium [20].

Catalytic and molecular properties of GluTR from different sources have been reported [15,21,22]. Crystal structure of *Methanopyrus kandleri* GluTR [23] supports the findings that the enzyme is a homodimer with an unusual V-shape. Each monomer contains three domains (I, II, III) connected by a spinal α-helix. The N-terminal region (responsible of the catalytic activity) includes domain I, that binds the glutamyl moiety of Glu-tRNA^{Glu} and forms the glutamyl-thioester intermediate, and domain II, the NADPH binding region. The C-terminal region (domain III) is responsible for the dimerization of GluTR [23].

Acidithiobacillus ferrooxidans, an industrial relevant acidophilic bacterium that participates in the bioleaching of minerals, uses ferrous ion (Fe^{2+}) and reduced sulfur compounds as electron donors for respiration [24]. Depending on metabolic requirements, cytochromes might reach up to 10% of the total proteins when grown in medium with Fe^{2+} [25]. Consequently, intracellular levels of heme are variable. Previous work from our laboratory revealed that cellular levels of *A. ferrooxidans* GluTR were substantially reduced upon increase in the intracellular heme content suggesting a possible role of the heme status in the function of GluTR [26]. To shed

Abbreviations: GluTR, Glutamyl-tRNA reductase; ALA, 5-aminolevulinic acid; GSA, glutamate-1-semi-aldehyde; GSA-AT, glutamate-1-semi-aldehyde 2,1-aminomutase; *o*-phen, *ortho*-phenanthroline.

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light into the effect of heme in the function of *A. ferrooxidans* GluTR, we analyzed the molecular and catalytic properties of the recombinant enzyme expressed in *Escherichia coli* under conditions that allowed us to control the intracellular level of heme. The data presented in this report led us to propose that in *A. ferrooxidans* a lower activity of GluTR upon binding of heme in combination with a reduced enzyme level upon increased intracellular heme [26] might broadly tune the function of GluTR and hence the biosynthesis of tetrapyrroles.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli strains (DH5α and BL21(DE3)) were grown aerobically at 37 °C in Luria–Bertani (LB) medium. Ampicillin was added at final concentration of 100 μg/ml if required. Media were supplemented with 100 μM *ortho*-phenanthroline (*o*-phen) [27] or 500 μM ALA for either low or high heme level [28].

2.2. Plasmid construction

DNA fragments encoding for GluTR357, GluTR392 and GluTR426 (Fig. 1A) were obtained by PCR amplification using genomic DNA as template and the following primers: GluTRforward 5′-CTCGAGTTGCAATCCTGTCCGGCAT-3′ and GluTR357reverse 5′-CTCGAGTTACTCCCGCCATTGCTG-3′, GluTR392reverse 5′-CTCGAGTTAGTCCTGCCCCTGATCC-3′, GluTR426reverse 5′-CTCGAGTTATTCGTTGGTCGCGTCC-3′ (underlined regions of all primers correspond to the XhoI recognition sequence). The resulting PCR products were digested with XhoI and ligated into appropriately digested plasmid pET15b, obtaining the plasmids pGluTR357Af, pGluTR392Af and pGluTR426Af. Upon expression, N-terminal His_6-tagged fusion proteins were obtained that facilitated further affinity chromatography purification. A DNA fragment encoding for the GluTR lacking the first twenty-one amino acids, was constructed by PCR amplification of genomic DNA with the following primers: NH_2GluTRforward 5′-CATATGACCACCATTTTCTGCTTCGG-3′ and NH_2GluTRreverse 5′-CTCGAGTCCTTCGGCGTCACTG-3′ (underlined regions represent the sequences recognized by NdeI and XhoI, respectively). PCR fragment was digested with NdeI and XhoI and ligated into the appropriately digested plasmid pET21b obtaining the plasmid pNH_2GluTRAf. A C-terminal His_6-tagged fusion protein was obtained and purified as mentioned above. Plasmid pGtrAfET, encoding GluTR, was described by Levicán et al. [29]. All plasmid constructs and mutants derived from PCR products were confirmed by DNA sequence analysis.

2.3. Protein expression and purification

Expression and purification of *A. ferrooxidans* GluTR variants (wild type and truncated mutants) was carried out as described [29]. His_6-tag was removed by overnight incubation at 4 °C with thrombin. The remaining His_6-tagged protein was separated by re-chromatography of the mixture on the Ni^{2+}-affinity resin. Purified proteins were dialyzed against buffer A (50 mM Hepes-NaOH pH 7.5, 150 mM NaCl, 20% (v/v) glycerol and 1 mM DTT). Protein concentration was determined using the standard dye-binding Bradford assay using bovine serum albumin as the protein standard [30].

2.4. UV–visible light absorption spectroscopy

UV–visible light spectra of purified recombinant wild type and truncated GluTR were recorded using Jasco V-550 spectrophotometer in 50 mM Tris–HCl pH 8.0. Samples were reduced by adding a few grains of sodium dithionite or oxidized by the addition of 0.5 mM $K_3Fe[CN]_6$. Heme concentration of purified proteins was determined by measuring the absorption at 419 nm (ε_{419} value of $1.915 \times 10^5\ M^{-1}\ cm^{-1}$ [31]) in alkaline pyridine (25% (vol/vol) pyridine in 0.1 M NaOH). For the determination of cellular heme concentration, cells harvested from 100 ml *E. coli* cultures grown under conditions to obtain high or low heme level were washed and resuspended in lysis buffer (50 mM Tris–HCl pH 7.5; 0.2% SDS), boiled for 10 min and the extract clarified by centrifugation at 20,000*g*, at 4 °C for 60 min. Heme concentration was determined as described above and expressed relative to protein concentration [30].

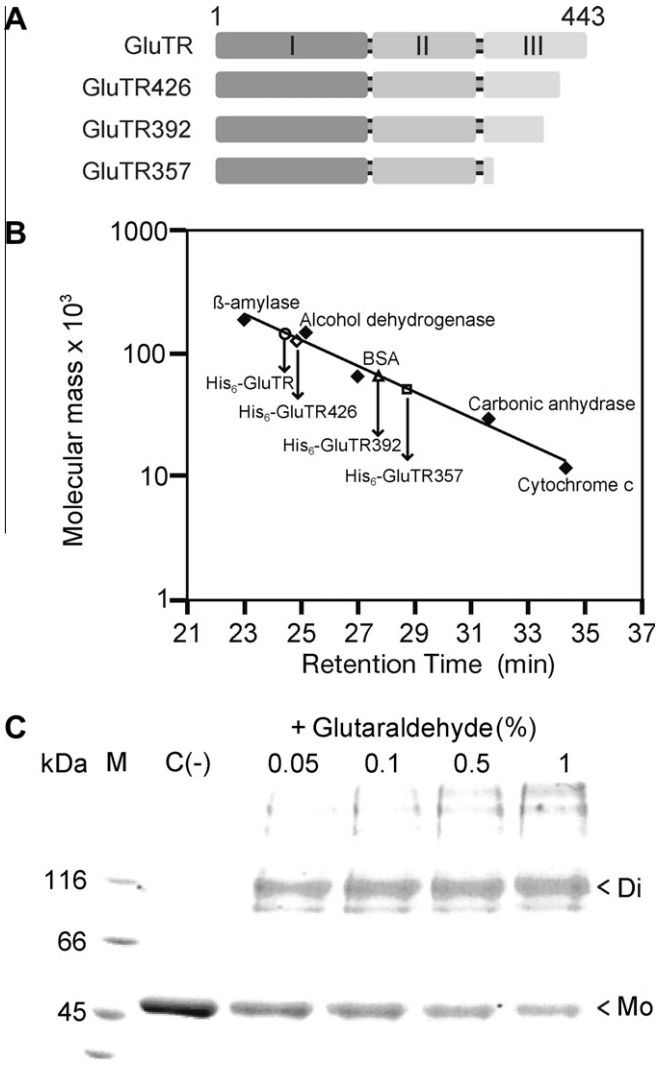

Fig. 1. GluTR expressed in *E. coli* is a dimeric enzyme. (A) Schematic representation of GluTR architecture, including catalytic domain (I, residues 1–190); NADPH binding domain (II, residues 198–350) and dimerization domain (III, residues 350–443) and the truncated mutants GluTR426 (residues 1–426), GluTR392 (residues 1–392) and GluTR357 (residues 1–357). (B) Relative native molecular mass of His_6-GluTR and the truncated variants by gel permeation on Superdex HR200. (C) Analysis by SDS–PAGE of glutaraldehyde cross-linked GluTR. C(−) control without glutaraldehyde; 0.05, 0.1, 0.5, 1% represent the concentration of glutaraldehyde used to cross-link the enzyme. D and Mo, dimeric and monomeric forms respectively. M, molecular weight marker.

2.5. GluTR assay

All procedures for the preparation of $tRNA_2^{Glu}$ by *in vitro* transcription were carried out as described [29]. GluTR activity was

measured by the [^{14}C]Glu-tRNA$_2^{Glu}$ depletion assay [15]. The substrate was prepared according to Shauer et al. [22] using recombinant *A. ferrooxidans* glutamyl-tRNA synthetase-1 [32].

2.6. Molecular mass determination

Analytical gel permeation chromatography was performed using Superdex 200 HR 10/30 column pre-equilibrated with 50 mM Tris–HCl pH 8.0, 150 mM NaCl. The column was previously calibrated with molecular weight standards (MW-GF200 Kit, Sigma) at flow rate of 0.5 ml/min. All proteins were applied to the column and run under identical conditions. MALDI–TOF molecular mass determinations were performed in a Microflex equipment (Brucker Daltonics) at the Facultad the Ciencias Químicas y Farmacéuticas, Universidad de Chile.

2.7. Cross-linking assays

Cross-linking of recombinant GluTR was performed as described [33] with slight modifications. Briefly GluTR (0.5 mg/ml) in phosphate buffer pH 7.5 was incubated at room temperature for 10 min with 0.05–1% glutaraldehyde. Subsequently, the protein sample was mixed with 5X sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer supplemented with 400 mM glycine and boiled for 5 min. Cross-linked samples were resolved on 8% SDS–PAGE.

3. Results

3.1. GluTR is a dimer that binds heme

N-terminal His$_6$-tagged GluTR was purified by Ni^{2+}-Sepharose affinity chromatography to near homogeneity as judged by SDS–PAGE analysis with an approximate molecular mass of 50 kDa. Polypeptide molecular mass was confirmed by MALDI-TOF mass spectrometry (50,489 ± 10 Da). After removal of the His$_6$-tag, *A. ferrooxidans* GluTR was still soluble. Relative molecular mass of native recombinant *A. ferrooxidans* GluTR, determined by gel permeation chromatography, was 148,000 ± 1000 Da, suggesting a trimeric protein (Fig. 1B). Cross-linking of *A. ferrooxidans* GluTR with glutaraldehyde led to the formation of a dimer with a molecular mass of 104,000 ± 1000 Da (Fig. 1C). These results led us to conclude that *A. ferrooxidans* GluTR is a dimer probably with an overall V shape that makes the protein to migrate slower in gel permeation chromatography, as with other reported GluTRs [15,22,23].

UV–visible absorbance spectrum of purified GluTR revealed the presence of heme as it showed Soret peak at 420 nm and a broad absorbance around 540 nm (Fig. 2A). Addition of sodium dithionite to reduce heme resulted in a slight shift of the Soret peak to 425 nm and the appearance of β and α bands at 530 and 558 nm respectively similar to the low spin heme complexes described for type *b* cytochromes [34]. Similar spectra were obtained for GluTR without His-tag or the His-tag at the C-terminus (data not shown). Pyridine hemochrome analysis confirmed the presence of type *b* heme bound to the recombinant GluTR, since α and β bands were at 556 and 526 nm, respectively, identical to myoglobin pyridine hemochrome (Fig. 2A, inset). Ratios of 1 heme bound per monomer or 1 heme per 16 monomers have been reported for *C. vibrioforme* [18] or *M. tuberculosis* [19] GluTR, respectively. Taking into account the discrepancies in the binding of heme to enzymes from different sources, we asked as to whether the binding of heme to *A. ferrooxidans* GluTR depends on the cellular availability of the cofactor. To address this question, before the induction with IPTG to overexpress *A. ferrooxidans* GluTR, the *E. coli* culture was supplemented with ALA [28] to increase the intracellular level of heme or with *o*-phen [27], a cell-permeative iron chelator, to reduce iron availability for heme biosynthesis. Expression of the enzyme was not affected by the culture supplementation with the chemicals. As expected, the cellular levels of heme increased in the presence of ALA or decreased by the addition of *o*-phen compared to the control (LB, without chemicals) (Fig. 2B). Consequently, ratios of 0.083, 0.25 or 0.5 molecules of heme bound per monomer (or 1 heme per 12, 4 or 2 monomers,

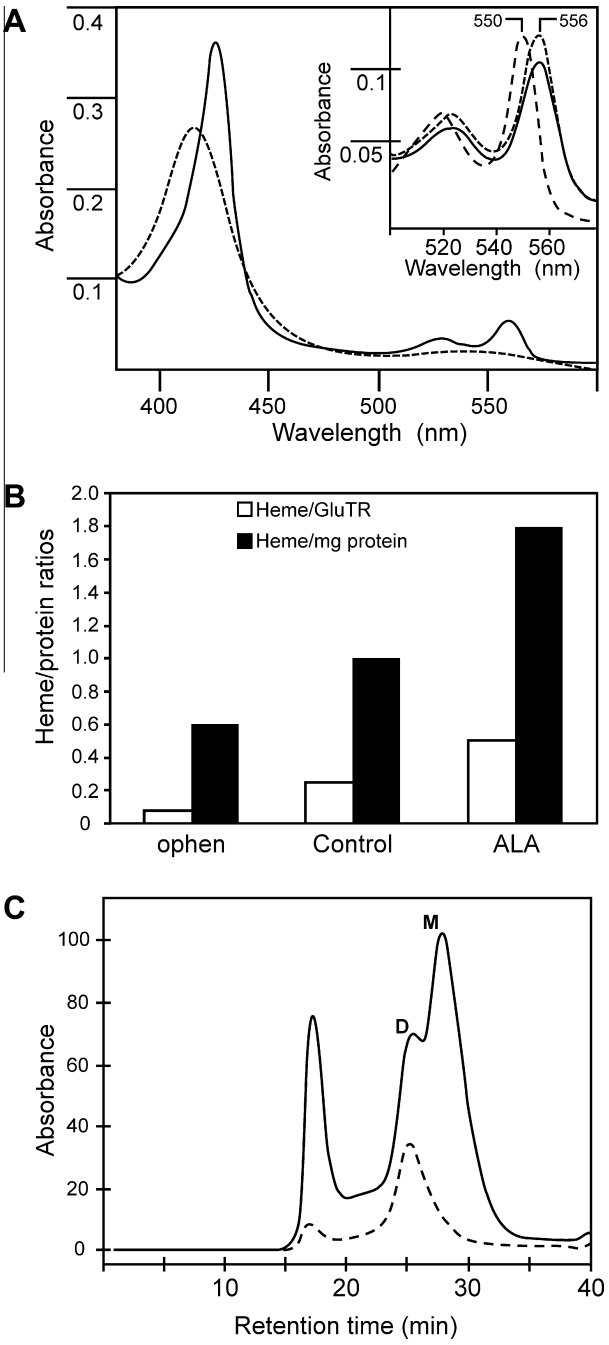

Fig. 2. Binding of heme to GluTR. (A) Absorption spectra of reduced (–) or oxidized (—) GluTR. Inset: spectra of the pyridine derivatives hemochromes from GluTR (—), myoglobin (–) and cytochrome *c* (. . .). 550 and 556 represent the maximum absortion of β bands. (B) Total cellular heme (micromoles/mg of protein) (■) and molecules of heme bound per GluTR monomer (□) expressed in *E. coli* grown in the presence of *o*-phen, ALA or control cells (without addition) are represented. (C) Retention time of heme (absorbance at 420 nm, dark line) and GluTR392 (absorbance at 280 nm gray line). M, indicates monomeric and D, the dimeric forms of GluTR.

respectively) were obtained at low, normal and high cellular heme content, respectively (Table 1, Fig. 2B).

3.2. Binding of heme inhibits the activity of GluTR

A slightly shorter chromatographic retention time was observed on gel permeation chromatography of GluTR with the highest content of heme (Table S1). Circular dichroism spectra revealed a slight increase (from 16 to 20%) in the α helix content as well as a decrease (from 33 to 29%) in the β sheet content of GluTR upon binding of heme. Thus, a conformational change of the dimer upon binding of heme might explain the difference in the elution behavior of the enzyme. Cross-linking experiments of each GluTR preparation revealed that binding of heme does not affect the dimerization of the enzyme (data not shown).

Purified *A. ferrooxidans* GluTR catalized Glu-tRNA hydrolysis [15] (Fig. 3A) as previously reported by Levicán et al. [29]. An inverse correlation between the catalytic efficiency of the enzyme and the amount of bound heme was observed. Catalytic efficiency of GluTR containing 0.083 heme per monomer was approximately threefold higher than GluTR containing 0.5 heme per monomer. V_{max} of the enzyme is the most affected kinetic parameter (five times decrease) upon increased binding of heme (Table 1). This data is in agreement with the inhibitory effect of heme reported for *C. vibrioforme* GluTR [18]. Dixon plot extrapolation [35] predicted that the enzyme without heme is twentyfold faster than the enzyme with one heme bound per dimer (data not shown). Thus, upon binding of heme to GluTR, a conformational change might take place that affects the catalytic efficiency of the enzyme.

3.3. Dimer formation is required for the efficient binding of heme

From the primary structure of *A. ferrooxidans* GluTR, a HRM sequence (*H*eme *R*egulative *M*otif; [36]) is predicted within the 21 amino acids at the N-terminus of the enzyme (Fig. S1). The $\Delta 21NH_2$GluTR variant, lacking the first 21 amino acids, which are not conserved in other GluTRs bound heme as well as the wild-type GluTR (data not shown). We then asked as to whether the dimerization of GluTR is required for the binding of heme. Based on the crystal structure of *M. kandleri* GluTR (pdb 1GPJ) [15], we constructed the truncated proteins GluTR357, 392 and 426, lacking 86, 51 and 17 residues, respectively, from the C terminus, responsible for the protein dimerization (Fig. 1A, Fig. S1). The three truncated mutants were obtained as soluble proteins and purified as the wild-type GluTR. Interestingly, all three mutants bound heme at different heme/protein ratios (Table 1). Gel permeation chromatography (Fig. 1B) revealed that GluTR357 (Fig. S2) and GluTR392 (Figs. 1B and 2C) were mostly monomers, although a small fraction of GluTR392 was dimer. GluTR426 that lacks 17 residues at the C-terminus was fully dimeric (Fig. 1B, Fig. S2). Strikingly, heme was bound predominantly to the dimeric forms of GluTR392 (Fig. 2C) and GluTR426 (Fig. S2). Heme bound to GluTR357 was very low and distributed in all enzymatic forms (Fig. S2). Taking together, these data suggested that heme is bound preferentially to the dimeric form GluTR. Surprisingly, none of the truncated proteins was active regardless of the oligomeric state (Fig. 3B). Thus, dimerization of GluTR is not sufficient for the enzyme to be active and the 17 residues at the extreme carboxy terminus are essential for this function.

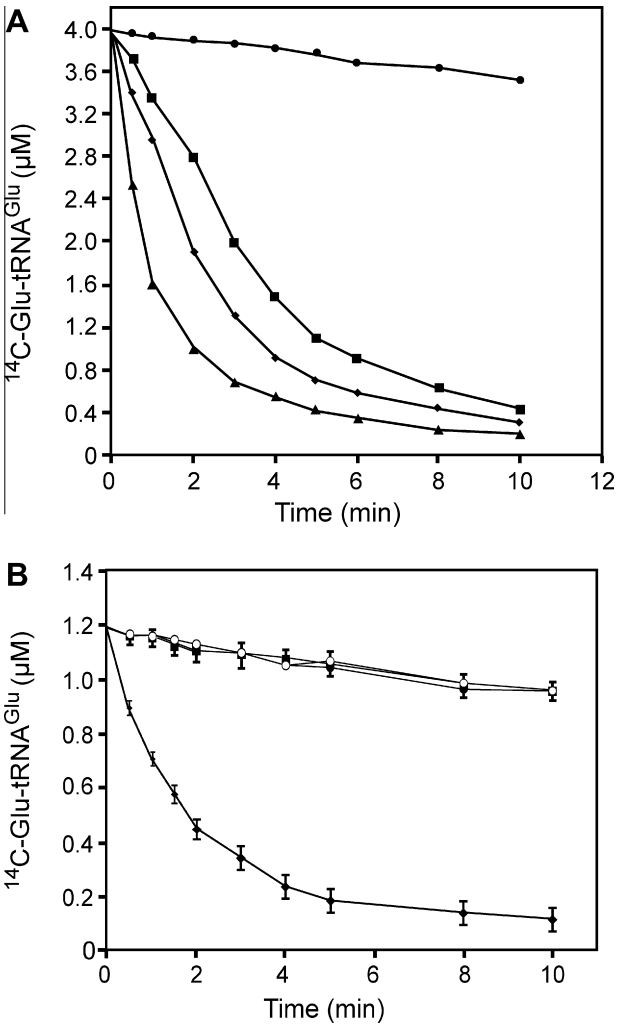

Fig. 3. GluTR enzymatic assay. (A) The depletion of [^{14}C]Glu-tRNA$_2^{Glu}$ (4 μM) by the enzymatic activity of 200 nM GluTRs with different heme/GluTR monomer ratios (■, 1:2), (♦, 1:4) and (▲, 1:12) was monitored. (B) Depletion of 1.2 μM [^{14}C]Glu-tRNA$_2^{Glu}$ by the enzymatic activity of 200 nM truncated (■, GluTR357; ▲, GluTR392; ○, GluTR426) or (♦), wild-type GluTR. Substrate hydrolysis in the absence of enzyme was used as background control (●).

Table 1
Binding of heme and kinetics parameters of GluTR.

Proteins	Heme/GluTR ratio[a]	V_{max}^{app}/K_m^{app} (min^{-1})	K_m^{app} ± S.E. (μM)	V_{max}^{app} ± S.E. (μM min^{-1})
Wild type full length proteins				
His_6-GluTR (*o*-phen)	1:12	0.63	17.2 ± 0.9	10.8 ± 0.4
His_6-GluTR (control)	1:4	0.34	13 ± 1	4.4 ± 0.3
His_6-GluTR (ALA)	1:2	0.22	11 ± 1	2.4 ± 0.3
Truncated mutant proteins				
His_6-GluTR426	1:4	ND	ND	ND
His_6-GluTR392	1:6	ND	ND	ND
His_6-GluTR357	1:10	ND	ND	ND

S.E., standard deviation.
ND, denotes no detectable activity.
[a] Heme/GluTR represents molecules of heme per GluTR monomer.

4. Discussion

GluTR is the first enzyme committed to heme biosynthesis by the C_5-pathway. Different mechanisms have been proposed for its regulation by heme. In *Salmonella thyphimurium* it has been proposed that GluTR stability is subjected to heme regulation [9,16,17]. A C170A mutant enzyme, lacking bound heme, was more resistant to degradation than the wild type [20]. Protein insolubility has hampered a detailed characterization of the effects of heme on GluTR [37]. Nevertheless, inhibition of GluTR from various sources by hemin, the Fe^{3+} analog of heme, has been described

[38,39] although in some cases it might be a non-specific effect [15,40]. Also heme bound to *C. vibrioforme* GluTR expressed in *E. coli* inhibited the enzymatic activity [18].

A. ferrooxidans GluTR bound heme *b*, at a maximum of 1 heme per dimer. Preferential binding to the dimeric form of GluTR was observed. Since no functional HRM (Heme Regulatory Motifs) was found in *A. ferrooxidans* GluTR, we predicted that amino acids from each subunit contribute to enhance the binding of heme to the dimer.

Although dimerization of GluTR occurred without the 17 amino acids at the carboxy terminus and the enzyme still bound heme, this enzyme form was not active. The entire carboxy terminus was required for the enzyme to be active. *S. thyphimurium* GluTR lacking the final six residues at the carboxy terminus was functional in an *in vivo* assay [20]. As the enzymatic activity was measured by the depletion of Glu-tRNA^{Glu}, we speculate that amino acids at the carboxy terminus might be implicated in the interaction of the enzyme with this substrate. This idea agrees with a model proposed for the interaction of GluTR with the substrates in which domain III interacts with the anticodon of the Glu-tRNA [23].

Type *a* and *b* non-covalent binding of heme to proteins are more suitable than type *c* (covalent) for signaling and regulatory functions due to the potential to sense variations in free heme concentrations [6]. We observed that *A. ferrooxidans* GluTR expressed in *E. coli* bound type *b* heme. The fraction of bound heme to GluTR was consistent with the cellular concentrations of the cofactor. Strikingly, as the level of heme bound increased, a negative effect on the enzymatic activity was observed. This observation is relevant to the physiology of *A. ferrooxidans* since the cellular levels of the cofactor are high and variable depending on the energy source used for the cells to proliferate [25]. Thus, we speculate that in *A. ferrooxidans* upon an increase in the intracellular heme concentration, an inhibition of the enzyme might be the first response that might be followed by the observed decreased in the levels of GluTR [26]. This proposed regulatory function could complement the effect of heme on the glutamyl-tRNA synthetase (GluRS1) from *A. ferrooxidans* [26], that provides the Glu-tRNA^{Glu} for the biosynthesis of heme as well as protein synthesis. This enzyme is subjected to reversible inactivation by heme and oxidizing agents [40].

Complex formation between GluTR and GSA-AT [41,42] as well as between GluTR and GluRS [43] have been proposed. These data tempted us to speculate that a complex between the three enzymes could channel the glutamate from Glu-tRNA^{Glu} to the biosynthesis of ALA and hence tetrapyrroles. Whether the binding of heme to GluTR alters the stability of the hypothetical complex formed by GluTR-GluRS-GSA-AT, and hence its function is an open question.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.013.

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