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**Lüer, C., Schauer, S., Virus, S., Schubert, W.-D., Heinz, D.W., Moser,**  
**J., Jahn, D.**  
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**> glutamyl-tRNA reductase**  
**> (2007) FEBS Journal, 274 (17), pp. 4609-4614.**

# **Glutamate Recognition and Hydride Transfer by *Escherichia coli***

## **Glutamyl-tRNA Reductase**

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Running Title: Substrate Recognition of GluTR

Subdivision: Enzymology

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### Abbreviations:

ALA, 5-aminolevulinic acid; DTT, 1,4-dithio-D,L-threitol; Tris, tris-(hydroxymethyl)-aminomethane; GluRS, glutamyl-tRNA synthetase; GluTR, glutamyl-tRNA reductase; Gln-tRNA<sup>Glu</sup>, glutamyl-tRNA<sup>Glu</sup>; Glu-tRNA<sup>Glu</sup>, glutamyl-tRNA<sup>Glu</sup>; GSA, glutamate-1-semialdehyde; GSA-AM, glutamate-1-semialdehyde 2,1-aminomutase; HEPES, N-(2-Hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)

### Enzymes:

glutamyl-tRNA reductase (GluTR) (EC 1.2.1.70)

glutamate-1-semialdehyde 2,1-aminomutase (GSA-AM) (EC 5.4.3.8)

glutamyl-tRNA synthetase (GluRS) (EC 6.1.1.17)

### Keywords:

substrate recognition; glutamyl-tRNA reductase; tetrapyrrole biosynthesis; glutamyl-tRNA; *Escherichia coli*

## Summary

The initial step of tetrapyrrole biosynthesis in *Escherichia coli* involves the NADPH-dependent reduction by glutamyl-tRNA reductase (GluTR) of tRNA-bound glutamate to glutamate-1-semialdehyde (GSA). We evaluated the contribution of the glutamate moiety of glutamyl-tRNA to substrate specificity *in vitro* using a range of substrates and enzyme variants. Unexpectedly, we found that tRNA<sup>Glu</sup> mischarged with glutamine (Gln-tRNA<sup>Glu</sup>) was a substrate for purified recombinant GluTR. Similarly unexpectedly, the substitution of amino acid residues involved in glutamate side chain binding (S109A, T49V, R52K) or in stabilizing the arginine 52 glutamate interaction (glutamate 54 and histidine 99) did not abrogate enzyme activity. Replacing glutamine 116 and glutamate 114, involved in glutamate-enzyme interaction near the aminoacyl bond to tRNA<sup>Glu</sup>, by leucine and lysine respectively, however, did abolish reductase activity. We thus propose that the ester bond between glutamate and tRNA<sup>Glu</sup> represents the crucial determinant for substrate recognition by GluTR, while the necessity for product release by a “back door” exit allows for a degree of structural variability in the recognition of the amino acid moiety. Analyzing the esterase activity, which occurred in the absence of NADPH, of GluTR variants using the substrate 4-nitrophenyl acetate confirmed the crucial role of cysteine 50 for thioester formation. Finally, the GluTR variant Q116L was observed to lack reductase activity while esterase activity was retained. Structure-based molecular modeling indicated that glutamine 116 may be crucial in positioning the nicotinamide group of NADPH to allow for productive hydride transfer to the substrate. Our data thus provide new information about the distinct function of active site residues of GluTR from *E. coli*.

## Introduction

5-Aminolevulinic acid (ALA), the precursor of all tetrapyrroles such as hemes and chlorophylls, is synthesized by two independent and unrelated routes. Animals, fungi and the  $\alpha$ -group of proteobacteria rely on the “Shemin pathway” [1-3], where ALA synthase, a pyridoxal-5'-phosphate-dependent enzyme synthesizes ALA from glycine and succinyl-CoA in one step [4]. In plants, green algae, archaea and most bacteria, by contrast, ALA is synthesized in two steps from glutamyl-tRNA<sup>Glu</sup> (Glu-tRNA<sup>Glu</sup>) [5-7]. First, glutamyl-tRNA reductase (GluTR) catalyzes the NADPH-dependent reduction of glutamyl-tRNA to highly reactive glutamate-1-semialdehyde (GSA). GluTR is thus one of a handful of enzymes using an aminoacylated tRNA in a biosynthetic pathway other than protein biosynthesis [8]. In a second step, GSA from GluTR is transaminated to ALA by the pyridoxal-5'-phosphate-dependent enzyme glutamate-1-semialdehyde 2,1-aminomutase (GSA-AM) [9, 10].

The catalytic mechanism of GluTR has been elucidated by different biochemical studies using recombinant protein from barley [11], *Methanopyrus kandleri* [12, 13], *E. coli* [14] and *Chlamydomonas reinhardtii* [15] which was ultimately supported by the solved crystal structure [13]. The conserved active site cysteine 50 (*E. coli* numbering) was found to nucleophilically attack the activated  $\alpha$ -carboxylate of glutamyl-tRNA creating a covalent thioacyl intermediate. This covalent intermediate has been detected through radioactive labeling studies for the GluTR from *E. coli* [14]. In a second step, covalently-bound glutamate is reduced to GSA by hydride transfer from NADPH. In the absence of NADPH, GluTR hydrolyzes the thioacyl intermediate releasing glutamate (figure 1). In contrast to thioester formation, hydride transfer from NADPH has not been analyzed in detail. The crystal structure of GluTR provides a “pre-active” view of the enzyme with respect to NADPH coordination. The NADPH-binding domain is rotated away from the catalytic domain such that the modeled NADPH is located at a distance of  $\sim 21$  Å from the active site [13]. Glutamyl-tRNA binding was proposed to induce the re-orientation of the NADPH-binding

domain enabling productive hydride transfer from NADPH. The GluTR variant G191D, affecting the second glycine of the NADPH-recognition motif GXGXXI of the NADPH-binding domain, is defective in hydride transfer, presumably because NADPH binding is inhibited [14].

The GluTR-tRNA interaction has been analyzed in detail [16]. Kinetic analysis of 51 *E. coli* Glu-tRNA<sup>Glu</sup> variant transcripts indicate that the unique tertiary core structure of tRNA<sup>Glu</sup> rather than anticodon or acceptor arm is essential for recognition by GluTR. Nevertheless, the aminoacyl bond linking the glutamyl-moiety to tRNA would need to be specifically recognized by GluTR as its localization is crucial for the ensuring enzyme-thioester bond. In the crystal structure of GluTR from *M. kandleri*, the active site is occupied by the inhibitor glutamycin, representing the 3'-end of the natural substrate [13]. Recognition of the glutamate-moiety is observed to involve an elaborate system of hydrogen bonds and in particular a bidentate salt bridge to the conserved arginine 52 (figure 2).

Based on the crystal structures of GluTR from *M. kandleri* and GSA-AM from *Synechococcus* sp., these enzymes were proposed to form a complex to allow the highly reactive reaction intermediate GSA to channel from one to the other protecting it from the surrounding medium [13]. An *in silico* model of the complex, places the active site entrance of each GSA-AM monomer opposite a “back door” in the catalytic domain of GluTR. Arginine 52, required for substrate binding, largely constitutes this back door. A lateral movement of its head group would open the door, allowing GSA to pass onto the active site of GSA-AM. Recently, the proposed complex between GluTR and GSA-AM and the resulting substrate channeling was verified by two research groups [17, 18].

Here we present a detailed analysis of determinants of the glutamate part of glutamyl-tRNA for substrate utilization. The contribution of the various active site residues in glutamate recognition and catalysis in particular hydride transfer are discussed.

## Results and discussion

### *Mischarged Gln-tRNA<sup>Glu</sup> is a substrate for E. coli GluTR*

The crystal-structure of GluTR from *M. kandleri* in complex with the substrate analogue glutamycin indicates the glutamate moiety of glutamyl-tRNA to be specifically recognized by a defined hydrogen-bond pattern and a bidentate salt bridge (figure 2) [13]. This salt bridge involves the positively charged guanidine group of arginine 52 (atoms N $\epsilon$  and N $\eta_1$ ) and the negatively charged substrate-carboxylate group (O $\epsilon_1$  and O $\epsilon_2$ ). It was inferred to be one of the major discriminating interactions between enzyme and substrate [13].

If this were strictly true, tRNA<sup>Glu</sup> misacylated with glutamine (Gln-tRNA<sup>Glu</sup>) should not represent a substrate for *E. coli* GluTR. Unexpectedly, we found that [<sup>14</sup>C]Gln-tRNA<sup>Glu</sup> was indeed a substrate for recombinant purified *E. coli* GluTR *in vitro*. HPLC analysis in combination with radiometric detection indicated that a novel compound eluting separately from glutamate and glutamine (figure 3) close to the position of GSA was produced. Due to the low abundance of the synthesized [<sup>14</sup>C] labeled substance we failed to determine its chemical nature via NMR or mass spectrometry. The fact that [<sup>14</sup>C]Gln-tRNA<sup>Glu</sup> is accepted as a substrate by GluTR implies that the recognition of glutamate by Arg52 and its surrounding hydrogen bonding network is not particularly strict.

### *Structural malleability of the glutamate recognition pocket*

The GluTR variants R52Q and R52K were generated to investigate the contribution of arginine 52 to substrate recognition. R52Q is found to be inactive while R52K retains 5% residual reductase activity *in vitro*. A positive charge should not be essential for substrate recognition as the uncharged glutamine bound to tRNA<sup>Glu</sup> was accepted as a substrate. Thus, the positive charge of arginine 52 may be required to stabilize the immediately neighboring residues glutamate 54 and histidine 99 as well as surrounding side chains. As, the position of the positive charge of lysine (N $\zeta$ ) is intermediate between that of the arginine N $\epsilon$  and N $\eta_1$ , coordination of glutamate 54 is possible, as well as coordination of the substrate (figure 2). To

establish the contribution of arginine 52 to the stabilization of the substrate recognition pocket, we substituted the neighboring residues histidine 99 and glutamate 54 by asparagine and lysine, respectively (figure 2). The variants H99N and E54K retained significant GluTR activity (table 1) corroborating earlier observations that the corresponding exchange H84N in GluTR of *M. kandleri* only partially reduced enzyme activity [12]. Apart from arginine 52-N $\eta$ , the side chains of serine 109 and threonine 49 also coordinate the carboxylate-O $\epsilon_1$  of the substrate glutamyl moiety. The GluTR variants S109A, T49V again showed decreased, but not completely abolished GluTR activity (table 1). Clearly, the region around arginine 52-N $\eta$  is malleable to some degree, allowing individual residues to be substituted without abrogating enzyme activity.

#### *Role of glutamine 116 during hydride transfer from NADPH*

In contrast to the well-studied glutamyl thioester formation during GluTR catalysis, the reduction of the enzyme-bound glutamate to GSA has not been analyzed in detail. The hydride ion is provided by NADPH. In the crystal structure of GluTR from *M. kandleri*, the NADPH-binding site within NADPH-binding domain was found to be located at a distance of  $\sim 21$  Å from the active site. The NADPH-binding domain was hence postulated to rotate, possibly in response to Glu-tRNA<sup>Glu</sup>-binding, placing NADPH near the substrate.

During a systematic screen of GluTR variants carrying mutations in the active site for reductase and esterase activity we identified a GluTR variant, Q116L, which no longer functions as a reductase but retains 30% of wildtype esterase activity. HPLC-analysis indicated that glutamate was actively released from the substrate, implying that Glu-tRNA<sup>Glu</sup> recognition was not impaired, but that GSA was not formed. Glutamine 116 thus clearly is not required for either substrate recognition or for the formation of the enzyme-bound intermediate. Subsequent hydride transfer was, however, completely abolished implicating participation of glutamine 116 in the NADPH-dependent reduction of the thioester intermediate. Rotating the NADPH-binding domain towards the catalytic domain *in silico*

places NADPH alongside the substrate analogue glutamycin, making hydride transfer from NADPH to the thioester possible. In this modeled closed state, glutamine 116 of the catalytic domain is similarly located in close proximity to the NADPH nicotinamide-moiety, indicating that although glutamine 116 is not involved in hydride transfer itself, its function may be to guide and position the nicotinamide to allow productive hydride transfer.

*GluTR esterase activity solely relies on residue cysteine 50*

To assess the contribution of active site amino acids to the cysteine 50-mediated transesterification reaction, we employed a recently published new method for determining the GluTR's esterase activity [19]. It is based on an established esterase assay, where the release of nitrophenol from an artificial substrate is detected spectrophotometrically. This allows 4-nitrophenyl acetate to be used as a minimal GluTR substrate, rather than hydrolyzing bulkier glutamyl-tRNA in the absence of NADPH. The small size of the acetate moiety permits transesterification to be analyzed independently of other GluTR substrate determinants. GluTR recognition of 4-nitrophenyl acetate most probably solely depends on the ester bond. Therefore, the role of individual active site amino acids to thioester intermediate formation can be elucidated using suitable GluTR variants. Except for variant C50S, no GluTR variant showed significantly decreased esterase activity with respect to the substrate 4-nitrophenyl acetate. The active site amino acids analyzed are thus clearly involved in recognition of the natural substrate Glu-tRNA<sup>Glu</sup>. Only cysteine 50 is required for thioester formation.

## Experimental procedures

### *Overexpression and purification of E. coli GluTR and glutamyl-tRNA synthetase (GluRS)*

Wildtype GluTR from *E. coli* and GluTR variants were produced as N-terminal His<sub>6</sub>-fusion proteins, renatured from inclusion bodies and purified as described [20]. Structural integrity of all mutant enzymes was verified using CD spectroscopy. Recombinant *E. coli* GluRS was purified to apparent homogeneity according to a published procedure [21].

### *Site-directed mutagenesis of E. coli hemA*

Individual residues of GluTR from *E. coli* were exchanged using the QuikChange<sup>TM</sup> kit (Stratagene, La Jolla, CA). The following oligonucleotides were employed - modified bases are underlined:

GTGCTGTCGACGTGCAACCAGACGGAAC TTTATCTT (R52Q),

CGTGGTGCTGTCGACGTGCAACAAAACGGAAC TTTA (R52K),

GGCGTGGTGCTGTCGGTGTGCAACC (T49V),

CAACCGCACGAAAC TTTATCTTAGCGTTG (E54K),

GACGCGGTTAGCAAATTTAATGCGTGTTGC (H99N),

CAGCGGCCTGGATGCACTGGTTCTG (S109A),

GGGAGCCGCTGATCCTCGGTCAGGTT (Q116L)

### *Aminoacylation of tRNA<sup>Glu</sup> with [<sup>14</sup>C]glutamate and [<sup>14</sup>C]glutamine*

The [<sup>14</sup>C]-labeled natural substrate for GluTR, [<sup>14</sup>C]Glu-tRNA<sup>Glu</sup>, was prepared as described before [14]. Misacylated [<sup>14</sup>C]Gln-tRNA<sup>Glu</sup> was prepared in 1 ml total volume containing 10 µg of tRNA<sup>Glu</sup> from *E. coli* and 1 mg of purified GluRS from *E. coli*. The mixture was incubated at 37°C for 60 min in 50 mM Na-HEPES, pH 7.0, containing 15 mM MgCl<sub>2</sub>, 25 mM KCl, 3 mM DTT, 4 mM ATP, and 24 µM [<sup>14</sup>C]Gln (6 µCi) with a specific activity of 254 mCi/mmol (9.40 GBq/mmol). The purity of [<sup>14</sup>C]Gln was analyzed by derivatization, high resolution HPLC and high-sensitivity detection by fluorescence according to the application instructions for amino acid analysis by pre-column derivatization using 9-

fluorenylmethoxycarbonyl chloride/1-aminoadamantane (Grom, Rottenburg-Hailfingen, Germany). No cross contamination with [ $^{14}\text{C}$ ]Glu was observed. Following the addition of 4 ml ice-cold 375 mM Na-acetate pH 5.2, the reaction mixture was extracted by phenol/chloroform and precipitated by ethanol [14]. The precipitate was dissolved in 30 mM Na-acetate pH 4.9, and the [ $^{14}\text{C}$ ]Gln-tRNA<sup>Glu</sup> was immediately used in the catalytic assays.

#### *GluTR catalytic assay*

Due to the low catalytic activity of GluTR variants and the use of misacylated tRNA substrate, the standard depletion assay for GluTR [14] was adapted. In most cases 5.7 nM to 30 nM purified, recombinant *E. coli* GluTR and 100 nM [ $^{14}\text{C}$ ]Glu-tRNA<sup>Glu</sup> or [ $^{14}\text{C}$ ]Gln-tRNA<sup>Glu</sup> were used per 15  $\mu\text{l}$  assay. In case of no detectable activity at this enzyme concentration, the enzyme concentration was increased up to 1  $\mu\text{M}$ . After different incubation times, the assay mixture was transferred onto Whatman 3MM filters and analyzed as described [14]. The formation of GSA was routinely quantified by HPLC [14]. Finally four enzyme concentrations were analyzed in triplicate. The activity was determined as the ratio of velocity and enzyme concentration [22]. The activity of the enzyme variants was calculated relative to that of the wildtype enzyme (100%).

#### *HPLC analysis of GluTR product formation*

Reaction products were analyzed on a Waters  $\mu\text{Bondapak}^{\text{TM}}$  C18 reversed phase column (3.9 x 150 mm, 125-Å pore size, 10- $\mu\text{m}$  particle diameter) as described previously [12].

#### *Esterase activity determination with 4-nitrophenyl acetate*

The substrate 4-nitrophenyl acetate was dissolved in acetonitrile at a concentration of 243 mM. The assay mixture (600  $\mu\text{l}$ ) contained 50 mM Na-HEPES, pH 8.1, 20 mM  $\text{MgCl}_2$ , 20 mM KCl, 3 mM DTT, 20% (w/v) glycerol and 5  $\mu\text{l}$  of a 192  $\mu\text{M}$  dilution of the 4-nitrophenyl acetate stock solution. The enzymatic hydrolysis of 4-nitrophenyl acetate was analyzed in a Jasco V-550 spectrophotometer at room temperature. The background resulting from spontaneous hydrolysis was subtracted by running an appropriate background control in a

reference cuvette. To start the reaction, 5 µl of enzyme were added after 1 min incubation and nitrophenol formation was monitored for 10 min at 400 nm.

### Acknowledgements

This investigation was supported by grants of the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. We thank Kalle Möbius, Dr. Lennart Randau and Denise Wätzlich for helpful discussion.

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## Figure legends

Fig. 1. Catalytic mechanism of GluTR with its natural substrate Glu-tRNA<sup>Glu</sup>. The reactive cysteine 50 sulfhydryl group of GluTR (E-SH) nucleophilically attacks the  $\alpha$ -carbonyl group of glutamyl-tRNA. This transesterification results in an enzyme-localized thioester intermediate while free tRNA (R<sub>1</sub>) is released. Hydride transfer from NADPH to the thioester-bound substrate subsequently produces GSA. In the absence of NADPH, the thioester intermediate is hydrolyzed by the intrinsic esterase activity of GluTR releasing glutamate. The artificial GluTR substrates (Gln-tRNA<sup>Glu</sup>, 4-nitrophenyl acetate) used in this study are indicated. R<sub>1</sub>: tRNA<sup>Glu</sup>, R<sub>2</sub>: 4-nitrophenol

Fig. 2. Schematic representation of the active site of GluTR from *E. coli*. The model is based on the crystal structure of GluTR from *M. kandleri* co-crystallized with the inhibitor glutamycin. Dotted lines represent observed hydrogen bonds between active site residues and glutamycin, double dashed lines indicate salt bridges. The nucleophilic attack of the substrate carbonyl carbon by cysteine 50 is marked by a dotted arrow. Solid arrows indicate amino acid substitutions (grey bonds).

Fig. 3. HPLC analysis of the conversion of [<sup>14</sup>C]Gln-tRNA<sup>Glu</sup> by recombinant *E. coli* GluTR. The compounds purified from the assay mixtures were separated with a flow rate of 0.75 ml/min on a Waters  $\mu$ Bondapak<sup>TM</sup> C<sub>18</sub> reversed phase column (3.9 x 150 mm, 125 Å pore size, 10  $\mu$ m partikel diameter) and the reaction products were identified by a radioactivity flow detector. A, HPLC separation of the compounds isolated from an assay mixture containing [<sup>14</sup>C]Gln-tRNA<sup>Glu</sup> without addition of recombinant *E. coli* GluTR as background control. B, with the addition of wildtype *E. coli* GluTR. Please note, that in panel B double the amount of [<sup>14</sup>C]Gln-tRNA<sup>Glu</sup> was employed. In addition to [<sup>14</sup>C]glutamine liberated from

tRNA<sup>Glu</sup> a novel compound in the assay with *E. coli* GluTR marked with an arrow, eluting close to the position of GSA, appeared.