Crystal Structure of a Non-Discriminating Glutamyl-tRNA Synthetase

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Running title: Structure of non-discriminating GluRS

Summary

Error-free protein biosynthesis depends on the reliable charging of each tRNA with its cognate amino acid. Many bacteria, however, lack a glutaminyl-tRNA^{Gln} synthetase. Instead, tRNA^{Gln} is initially mischarged with glutamate by a non-discriminating glutamyl-tRNA synthetase (ND-GluRS). Such non-discriminating GluRS thus charge both tRNA^{Glu} and tRNA^{Gln} with glutamate, while discriminating GluRS (D-GluRS), found in some bacteria and all eukaryotes, exclusively generate Glu-tRNA^{Glu}. Here we present the crystal structure of ND-GluRS from *Thermosynechococcus elongatus* (ND-GluRS$_{Tel}$) in complex with glutamate at a resolution of 2.45 Å. Structurally the enzyme shares the overall architecture of the discriminating GluRS from *Thermus thermophilus* (D-GluRS$_{Tth}$). We confirm experimentally that GluRS$_{Tel}$ is non-discriminating. Kinetic parameters for synthesis of Glu-tRNA^{Glu} and of Glu-tRNA^{Gln} were determined. Anticodons of tRNA^{Glu} (^{34}C/UUC^{36}) and tRNA^{Gln} (^{34}C/UUG^{36}) differ only in base 36. Discriminating GluRS$_{Tth}$ specifically recognizes the pyrimidine base of C36 through Arg358. In non-discriminating GluRS$_{Tel}$ this arginine is replaced by glycine (Gly366) allowing both cytosine and the bulkier purine base G36 of tRNA^{Gln} to be tolerated. Most other non-discriminating GluRS share this structural feature, leading to relaxed substrate specificity.

Introduction

The faithful translation of genetic information requires that transfer RNA (tRNA) is reliably loaded with its cognate amino acid before reaching the ribosome during protein biosynthesis. This is ensured by a set of aminoacyl-tRNA synthetases (AARS) each of which catalyzes the aminoacylation of a specific tRNA. Based on their distinct ATP-binding cores, AARS fall into two classes of 10 enzymes each.[1] Active sites of class I enzymes (including GluRS and GlnRS) involve a parallel β-sheet Rossmann fold with two conserved amino acid motifs for ATP binding. Class II enzymes (AspRS, AsnRS and others) exhibit a central seven-stranded, antiparallel β-sheet with three conserved motifs.[1]

tRNA aminoacylation is a two-step event. In the first step, the AARS activates the amino acid using ATP and Mg^{2+} to yield an enzyme-bound, high-energy aminoacyl adenylate intermediate. In the second step, the aminoacyl moiety is transferred to the 3'-terminal adenosine of the cognate tRNA. In GluRS, GlnRS and ArgRS, the first step occurs only in the presence of the tRNA.[2]

AARS reliably discriminate between different tRNAs, such that the 20 proteinogenic amino acid residues generally require a corresponding number of tRNAs and AARS.[3] Numerous organisms, however, express fewer than these 20 AARS.[4] In particular, glutaminyl-tRNA, asparaginyl-tRNA[5] and cysteinyl-tRNA[6] synthetases may be affected. Thus all archaea[7], most bacteria, as well as mitochondria and chloroplasts[8] lack glutaminyl-tRNA synthetase (GlnRS). In exchange, a non-discriminating glutamyl-tRNA synthetase (ND-GluRS) charges both tRNA^{Glu} and tRNA^{Gln} with glutamate (Scheme 1).[9] The resulting misacylated Glu-tRNA^{Gln} is then converted to Gln-tRNA^{Gln} by a tRNA-dependent amidotransferase (AdT) through the addition of amide to the glutamate. The indirect synthesis known as the transamidation pathway is the more ancient way of producing Gln-tRNA^{Gln}.[10]

Scheme 1

The anticodon-binding domains of bacterial and archaeal/eukaryotic GluRS differ structurally. While the former has an all α-helical topology, the secondary structure of the

latter exclusively involves β-strands.[11] Sequence comparisons and the all-β anticodon-binding domain indicate that GlnRS evolved from GluRS in eukaryotes shortly after the archaea/eukarya divide.[12] Some bacterial species such as *Thermus*, *Deinococcus* and proteobacteria of the β- and γ-families then presumably acquired GlnRS by horizontal gene transfer.[13]

Here we present the first crystal structure of a non-discriminating GluRS from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (ND-GluRS$_{Tel}$) at 2.45 Å resolution. The principal structural feature distinguishing ND-GluRS$_{Tel}$ from discriminating GluRS from *Thermus thermophilus* (D-GluRS$_{Tth}$)[11] is the substitution of an arginine by glycine in the anticodon-binding domains, allowing the bulky base G36 of the tRNA^{Gln}-anticodon to be accommodated. Moreover, we present catalytic constants for charging Glu-tRNA^{Glu} and Glu-tRNA^{Gln} confirming GluRS$_{Tel}$ to be non-discriminating.

Results

GluRS from *Thermosynechococcus elongatus* is non-discriminating

The genome[14] of the thermophilic cyanobacterium *Thermosynechococcus elongatus* does not include a gene encoding a glutaminyl-tRNA synthetase (GlnRS).[15] Instead, the genes *gatC*, *gatA* and *gatB* for the heterotrimeric amidotransferase Glu-Adt (or GatCAB)[16] are present.[15] Glutamyl-tRNA synthetase of *T. elongatus* (GluRS$_{Tel}$) is thus presumably non-discriminating, acylating both tRNA^{Glu} and tRNA^{Gln} followed by the conversion of misacylated Glu-tRNA^{Gln} to Gln-tRNA^{Gln} by GatCAB.

To unambiguously demonstrate the non-discriminating character of GluRS$_{Tel}$, recombinant purified enzyme and mature tRNA^{Glu} and tRNA^{Gln} purified from *T. elongatus* extracts[17] were used in aminoacylation assays. Northern dot-blot analyses confirm the complete separation of the extract-derived tRNAs (data not shown). Glutamylation of tRNA^{Glu} and tRNA^{Gln} were quantified by iterative Lineweaver-Burk analysis over a substrate range from 0.2 to 7 μM (Table 1). The kinetic data for misacylation of tRNA^{Gln} by GluRS$_{Tel}$ were found to be K_m = 3.7 μM and k_{cat} = 0.036 s^{-1} yielding a k_{cat}/K_m = 9.7 s^{-1}mM^{-1}. Corresponding values for tRNA^{Glu} are K_m = 0.79 μM, k_{cat} = 0.1 s^{-1} and k_{cat}/K_m = 126 s^{-1}mM^{-1}. Glutamylation of tRNA^{Glu} is thus 13-fold more efficient (k_{cat}/K_m) than that of tRNA^{Gln}.

By comparison, the discriminating GluRS from *E. coli* is virtually unable to misacylate the noncognate tRNA^{Gln} with a K_m of 190 μM and a k_{cat} of 7 x 10^{-5} s^{-1} yielding a k_{cat}/K_m of 10^{-8} s^{-1}mM^{-1}.[18] Therefore, tRNA^{Gln} does not serve as a natural substrate for D-GluRS. A similar mischarging reaction was analyzed for the aminoacylation of tRNA^{Asn} by non-discriminating aspartyl-tRNA synthetase (AspRS) of *Thermus thermophilus*.[19] This bacterium does not only possess discriminating AspRS (AspRS1) and AsnRS, but also a non-discriminating AspRS (AspRS2). The k_{cat}/K_m for aminoacylation of tRNA^{Asn} by the discriminating AspRS1 was 2250-fold lower than for charging tRNA^{Asp}, while the non-discriminating AspRS2 showed only a 2-fold difference in efficiency.[19]

Table 1

Structure of ND-GluRS$_{Tel}$

The crystal structure of ND-GluRS$_{Tel}$ (54.4 kDa) was solved by molecular replacement using discriminating GluRS from *Thermus thermophilus* (D-GluRS$_{Tth}$; PDB-code 1GLN) which shares a sequence identity of 37% to GluRS$_{Tel}$ as search model, and refined to a resolution of 2.45 Å. The asymmetric unit contains two molecules. All 485 amino acids of monomer A are well resolved, while a further four residues of the C-terminal His$_6$-tag are visible in the electron density of monomer B. Data collection and refinement statistics are summarized in Table 2.

Table 2

ND-GluRS$_{Tel}$ is an elongated, slightly curved molecule, 110 Å in length and 35-40 Å in width.

Fig. 1&2

The modular protein consists of five domains (Figures 1 and 2(a)). The N-terminal, catalytic domain (green in Fig. 1) has a dinucleotide-binding or Rossmann fold characterized by a central 5-stranded, parallel β-sheet, flanked by α-helices. A deep pocket accommodates the active site, including binding sites for glutamate and the acceptor end of tRNA. The conserved ATP-binding motifs, HIGT and KLSKR, respectively located at the N-terminal end of α-helix αA and in a loop between β-strand β11 and helix αI, form part of the active site pocket. The acceptor-binding or connective-polypeptide (CP) domain is sequentially inserted into the catalytic domain, dividing the latter into two subdomains. The acceptor-binding domain, comprising three α-helices and two anti-parallel β-sheet of four and two strands respectively, causes the helical tRNA acceptor end to change its conformation during binding to allow the 3'-end to fit into the active site[20]. The two N-terminal domains are followed by three α-helical domains: The stem-contact (SC) domain (4 α-helices) and two anticodon-binding domains, a three-helix bundle and a six-helix cage, respectively. The crystallographically independent monomers A and B are structurally largely identical. Their modular architecture, however, allows monomer A to bend slightly more than monomer B.

The difference leads to a root mean square deviation (r.m.s.d.) of 0.9 Å for the C_α atoms of both molecules.

A Zn^{2+} binding site involving the so-called SWIM motif Cys-x-Cys-x_{24}-Cys-x-His has been identified in GluRS from *Escherichia coli* by extended X-ray absorption fine structure (EXAFS) analysis and site-directed mutagenesis studies[21; 22]. It serves to stabilize a segment of the CP-domain responsible for interacting and positioning the tRNA acceptor end correctly in the active site. Though ND-GluRS$_{Tel}$ bears a similar motif (Cys98-x-Cys100-x_{24}-His125-x-His127) and 250 μM zinc acetate was added to the crystallization drops, Zn^{2+} was not observed to bind. Instead the side chains of Cys98, Cys100 and His125 are in mutual van-der-Waals contact while His127 is structurally removed and points away from the other potential ligands. Aminoacylation assays show that ND-GluRS$_{Tel}$ does not require zinc whereas zinc is essential for the activity of GluRS from *E. coli*[21]. This is consistent with the rapid rate of evolution indicated by phylogenetic studies that revealed frequent differences of the putative zinc-binding sites between closely related taxons[22].

Glutamate recognition

In ND-GluRS$_{Tel}$ monomer A, a glutamate substrate molecule is bound within a positively charged pocket of the active site (Figure 3(a)). Its γ-carboxyl group forms two salt bridges to the guanidinium groups of Arg6 and Arg210 and a hydrogen bond to the Tyr192 hydroxyl group. The α-carboxyl group is involved in a hydrogen bond or salt bridge to atom His214-$N_{\delta 1}$. The amino acid specificity of GluRS is thus ensured by the glutamate binding site recognizing substrates of suitable size and charge distribution.

Fig. 3

Discussion

Structural differences between ND-GluRS$_{Tel}$ and D-GluRS$_{Tth}$

As indicated by the sequence identity of 37%, ND-GluRS$_{Tel}$ is structurally similar to D-GluRS$_{Tth}$ (Figure 2). The three N-terminal domains show a particularly high degree of conservation (sequence identity of 44%) with extended stretches of conserved residues being especially apparent in the catalytic domain. Correspondingly, these domains of ND-GluRS$_{Tel}$ and D-GluRS$_{Tth}$ align particularly well. A sequence identity of only 25% in the anticodon-binding domains results in distinct differences reflecting their structural rather than functional role[23]. Thus, only individual residues of the hydrophobic core and the C-terminus of αS are conserved. Many α-helices are displaced while some loops adopt distinctly diverging conformations. The superposition of ND-GluRS$_{Tel}$ monomer A on apo (PDB-code 1GLN)[11] and on tRNA-bound D-GluRS$_{Tth}$ (1N77)[2] yield r.m.s.d. values of 3.3 (2.9 for monomer B) and 3.0 (2.9) Å for 459 common C$_\alpha$ atoms. These deviations primarily result from subtle domain reorientations in different GluRS molecules.

Insertions/deletions in primary structure give rise to the most prominent structural differences between ND-GluRS$_{Tel}$ and D-GluRS$_{Tth}$. The first, by sequence (Δ1 in Figure 2(a)), involves eight residues inserted between β3 and αC of the catalytic domain, and mainly occurs in γ-proteobacteria and spirochaetes GluRS sequences. Δ1 creates a bulge in D-GluRS$_{Tth}$ not involved in tRNA-binding or catalysis and its absence in ND-GluRS$_{Tel}$ presumably has no functional implications. The second insertion, Δ2, involves six amino acids in the acceptor-binding domain present in all bacterial GluRS enzymes (including ND-GluRS$_{Tel}$) but absent merely in the discriminating enzymes from the *Deinococcus*/*Thermus* species (D-GluRS$_{Tth}$). Δ2 elongates α-helix αD C-terminally by one turn and lengthens the adjoining loop. Δ3, an insertion of five residues exclusively observed in cyanobacterial GluRS, serves to lengthen the loop between β8 (CP domain) and αG (catalytic domain) in ND-GluRS$_{Tel}$ forcing αF to shift by ~7 Å compared to D-GluRS$_{Tth}$. Neither Δ3 nor αF are involved in tRNA binding or

Fig. 2

catalysis. Two minor insertions ($\Delta4$, $\Delta5$) located in the anticodon-binding domains again extend two loops on the side of the enzyme not involved in tRNA-binding, while $\Delta6$ elongates the C-terminus of ND-GluRS$_{Tel}$ by six amino acids.

Substrate recognition in the active site

A model of tRNA^{Glu}-bound ND-GluRS$_{Tel}$ was obtained by superimposing tRNA^{Glu}-bound D-GluRS$_{Tth}$[2] on ND-GluRS$_{Tel}$. In this model, the elongated protein forms a continuous interaction interface with one side of the L-shaped tRNA extending from the anticodon loop to the acceptor end.

The acceptor-end of tRNA^{Glu} structurally matches its binding site created by the catalytic and

acceptor-binding domains (Figure 3(b)). A protrusion of the CP domain unwinds the helical acceptor-end of the unbound tRNA inducing the 3'-end to adopt a hairpin conformation required to fill the active site. As observed in D-GluRS$_{Tth}$, C74 is presumably accommodated by a pocket in the CP domain, while A73, C75 and A76 stack upon His214 and Tyr192.

Insertion $\Delta2$ (see above) increases the size of the protrusion separating the tRNA 5' and 3'-ends in ND-GluRS$_{Tel}$ compared to D-GluRS$_{Tth}$. This extension is common to all non-discriminating, bacterial GluRS, but not the discriminating *Deinococcus/Thermus* enzymes. The insertion would thus appear to be required for tRNA^{Gln} but not tRNA^{Glu}-binding.

In D-GluRS$_{Tth}$ ATP initially binds to a 'non-productive' subsite spatially removed from the second substrate glutamate.[2] Upon tRNA-binding, ATP moves to the 'productive' subsite reacting with glutamate to yield the intermediate glutamyl-AMP, which aminoacylates the tRNA. Most active-site residues are conserved in ND-GluRS$_{Tel}$ indicating that the described mechanism is similarly conserved.

Recognition of tRNA^{Glu} and tRNA^{Gln}

In the complex of tRNA^{Glu}/GluRS$_{Tth}$, the anticodon loop of tRNA^{Glu} adopts a U-turn structure while its minor groove interacts with the two anticodon-binding domains[24]. Our model indicates that this occurs similarly in ND-GluRS$_{Tel}$. The anticodon-binding domain of ND-

GluRS$_{Tel}$ forms a cavity similar to the one accommodating the stacked anticodon nucleotides

C34 and U35 in D-GluRS$_{Tth}$ (Figure 4). These bases are thus recognized by the helix-loop-

Fig. 4   helix structure of αM, αM-αN and αN (residues 437-458 in ND-GluRS$_{Tel}$). C34 would

laterally interact with the hydrophobic side chains of Met441, Leu457 and Met458 while

atoms O_2 and N_3 form hydrogen bonds to the side-chain guanidinium group of Arg445 and the

main-chain amide group of Leu447, respectively. In *E. coli*, a modified uridine at position 34

(5-methylaminomethyl-2-thiouridine, mnm^{5}s^{2}U) is a major identity element for tRNA^{Glu}

recognition by GluRS[25]. Though the modification of this base in tRNA^{Glu} from *T. elongatus*

has not been analyzed, a modified mnm^{5}s^{2}U^{34} could feasibly be accommodated. The 2-

thiocarbonyl moiety could form a hydrogen bond to Arg445, while the 5-methylaminomethyl

group facing away from the protein would not incur any steric clashes. Our model indicates

that U35 is presumably recognized through hydrogen bonds U35-O_2—Gly454-O, U35-N_3—

Gly454-N, and U35-O_2—Val452-O.

The anticodons of tRNA^{Glu} (^{34}C/UUC^{36}) and tRNA^{Gln} (^{34}C/UUG^{36}) differ only with respect to

base 36. Discrimination and non-discrimination in GluRS must, therefore, be linked to

differences in recognition of this nucleotide. In discriminating GluRS$_{Tth}$ [19], the guanidinium

side chain Arg358 of the first anticodon-binding domain specifically recognizes the

pyrimidine base C36 through hydrogen bonds to O_2 and N_3 (Figure 4(d)). This arginine is

replaced by glycine (G366) in non-discriminating GluRS$_{Tel}$, abrogating recognition of C36 of

tRNA^{Glu} and creating a cavity that allows the larger purine base G36 of tRNA^{Gln} to be easily

accommodated. While the binding of tRNA^{Gln} to GlnRS requires a rearrangement of the three

anticodon bases allowing each to bind to a separate binding pocket[26], the binding to ND-

GluRS$_{Tel}$, by contrast, assumes the anticodon bases of tRNA^{Gln} to stack as documented for

tRNA^{Glu}. Since this is presumably the structure of tRNA^{Gln} in solution, a rearrangement of

nucleotides would not be required during binding.

Discrimination versus non-discrimination by GluRS

Evolutionarily, non-discrimination of tRNA^{Glu} and tRNA^{Gln} by GluRS is more ancient, while discrimination appears first to have occurred in eukaryotes following the divergence of GlnRS from non-discriminating GluRS[13]. The transfer of GlnRS to individual bacteria allowed the GluRS to become discriminating by additionally distinguishing anticodon base 36 through an additional binding site in the first anticodon-binding domain. Non-discrimination by GluRS thus represents the standard situation, while discrimination required a dedicated fine-tuning of the enzyme to ensure that the previously acceptable G34 of tRNA^{Gln} is reliably rejected.

Sequence alignments of non-discriminating GluRS indicate that Gly366 of ND-GluRS$_{Tel}$ is conserved in *Caulobacter crescentus*, *Fusobacterium nucleatum* and GluRS1 of *Thermoanaerobacter tengcongensis* (incorrectly annotated as GluRS2 in UniProt), replaced by serine in cyanobacterial GluRS, by glutamine/glutamate in firmicute (e.g. *Bacillus*, *Lactobacillus* or *Mycoplasma*) GluRS. The fact that a glycine or glutamine in this position is not sufficient to allow discrimination is illustrated by the loss of discrimination in the mutant R358Q of D-GluRS$_{Tth}$.[24]

Interestingly, an arginine is observed at the position corresponding to Gly366 of ND-GluRS$_{Tel}$ (or Arg358 of D-GluRS$_{Tth}$) in actinobacteria, spirchaetes and chlamydiae. However, these species lack a gene for GlnRS but do carry *gatCAB*[15], implying their GluRS to be non-discriminating. An arginine at this position in itself therefore does not suffice to ensure discrimination. In tRNA-free D-GluRS$_{Tth}$ Arg358 is stabilized by a salt bridge to Glu443. Upon tRNA-binding, this interaction is disrupted. Glu443 interacts with the tRNA backbone while Arg358 forms a stacking interaction with Pro357 and a hydrogen bond to Leu354-O. The intricate stabilization of arginine in D-GluRS$_{Tth}$ indicates this to be crucial to discrimination. This inference is supported by the fact that non-discriminating GluRS with an arginine in position 366 (D-GluRS$_{Tel}$) never possess a glutamate at position 443.

Yet another variation is observed in some proteobacteria such as *Helicobacter*, *Rickettsia* or *Bartonella*. These species express both a non-discriminating GluRS1 and a discriminating GluRS2.[15] In all corresponding GluRS2, an arginine is observed at the critical discrimination position. GluRS1 all feature an asparagine or glutamate at this position.

Comparison with non-discriminating aspartyl-tRNA synthetase

The anticodons of tRNA^{Asp} (^{34}GUC^{36}) and tRNA^{Asn} (^{34}GUU^{36}) similarly differ in the third base, as described for tRNA^{Glu} and tRNA^{Gln} (see above). Discrimination in AspRS therefore involves two pyrimidines, cytosine and uracil, that differ only in the functional group at atom C_4. In these class-II synthetases, neither related in sequence nor in structure to GluRS[27], recognition of cognate tRNA exclusively involves the anticodon-binding domain. Discriminating and non-discriminating AspRS mainly differ with respect to the so-called L1-loop involved in recognizing the third anticodon nucleotide[28]. This loop is mostly five to seven residues longer in discriminating AspRS. A conserved proline in this loop was also found to be essential for mischarging AspRS[28; 29], while a histidine and a glycine (His31 and Gly83 in *Pseudomonas aeruginosa*) were identified to be conserved in non-discriminating, bacterial AspRS[30].

Conclusions

Comparing the molecular basis of discrimination of GluRS and AspRS indicates the former to be less complex. Essentially the introduction of an arginine in position 366 and a glutamate in position 443 appear sufficient to ensure discrimination. No insertions, deletions or large conformational changes are required. Hence, the observation that enzymes of *Thermus/Deinococcus* and proteobacteria are individually more closely related to non-discriminating GluRS than to each other may indicate that this principle of GluRS discrimination may have evolved more than once.

Materials and Methods

Cloning, production and purification of GluRS

The gene for GluRS (gltX) of *Thermosynechococcus elongatus* strain BP-1 was amplified from genomic DNA and cloned into the *Nde*I/*XhoH*I sites of the *Escherichia coli* expression vector pET29 (Novagen). The resulting plasmid encodes ND-GluRS$_{Tel}$ with a native N-terminus and a C-terminal His-tag. Transformed *E. coli* BL21 CodonPlus cells (Stratagene) were cultivated at 37°C to OD$_{600}$ of 0.6 in LB-medium with 30 μg/ml kanamycin and 50 μg/ml chloramphenicol. The temperature was lowered to 20°C, protein expression induced with 100 μM isopropyl-ß-D-thiogalactopyranoside, and the cells cultivated for 20 h. Cells were centrifuged, disrupted by French press and cell debris removed by centrifugation at 4°C. ND-GluRS$_{Tel}$ was isolated from the soluble fraction by Ni-NTA affinity chromatography and purified by anion exchange chromatography (MonoQ HR 10/10, GE Healthcare) using a linear gradient 10 to 400 mM NaCl in 20 column volumes in 20 mM Hepes pH 7.9, 2 mM DTT and 250 μM MgCl$_2$. GluRS fractions were pooled and concentrated by a 10 kDa cut-off Vivaspin centrifugal concentrator (Vivascience) and further purified using a Superdex 75 26/60 gel filtration column (GE Healthcare) in 20 mM Hepes pH 7.9, 20 mM NaCl, 10 mM DTT and 250 μM MgCl$_2$ at a flow rate of 2 ml/min. Purified ND-GluRS$_{Tel}$ was concentrated to 3 mg/ml. The overall yield was ~10 mg protein / L of bacterial culture. Protein integrity and purity were verified by SDS-PAGE, N-terminal protein sequencing, dynamic light scattering and mass-spectrometry.

Cultivation of *T. elongatus* and preparation of cell free extracts

T. elongatus BP1 cells were cultivated in a 5-liter fermenter at 55°C under continuous illumination from fluorescent white lamps (~80 μmol of photons·m^{-2}s^{-1}). The cells were grown in a DTN-Medium supplemented with micronutrients in an CO$_2$-enriched atmosphere of 10 % to an OD$_{800}$ of 2.0 as described before[31]. After harvesting by centrifugation 1.5 g wet cell mass / l culture were obtained. A total of 17 g cells were washed in buffer containing 50

mM Tris-Cl pH 7.5, 10 mM Mg acetate, 3 mM DTT. 1.5 mg/l RNaseOut (Invitrogen, Karlsruhe, Germany) in the same buffer were added. The cells were ruptured via sonification (Bandelin HD 2070, 0.5 s sound, 0.5 s paused, 70 % amplitude) using a sonotrode (MS73 tip, Bandelin, Berlin, Germany). The cell free extracts were ultracentrifuged at 4°C and 100,000 g. The supernatant was processed further.

Isolation of tRNA^Glu and tRNA^Gln from *T. elongatus*

Mature tRNA^Glu and tRNA^Gln from *T. elongatus* were obtained by acidic phenol extraction as described.[32] The tRNA extracted from 200 mg of total RNA was purified by anion exchange chromatography using the Qiagen Q500 Maxi Kit (Qiagen, Hilden, Germany). Total tRNA containing 9 mg was then deacylated by incubation at 37°C for 1 h in the presence of 200 mM Tris-acetate pH 9.0 and the deacylated tRNAs were recovered by ethanol precipitation[33].

tRNA purification

Mature tRNA^Gln and tRNA^Glu from *T. elongatus* were isolated by an improved solid-phase DNA probe method ("chaplet" column chromatography)[34]. 150 μg of 5'-biotinylated, gel-purified DNA oligonucleotides, complementary to the target tRNA was bound to streptavidin beads (Pierce) in 10 mM Tris-HCl pH 7.5. tRNA: 5'-biotin-CCCGCTGCCTAACCGCTTG-GCGACACCCCA-3' for tRNA^Gln and 5'-biotin-GGAGGTGTCCTAGGCCACTAGACGA-TGGGGGC-3' for tRNA^Glu (Biomers·net GmbH, Ulm, Germany). Beads were equilibrated in 6x NTE solution (20x NTE: 4 M NaCl, 0.1 M Tris-HCl pH 7.5, 50 mM EDTA, 5 mM β-mercaptoethanol), 10 mg unfractionated *T. elongatus* tRNA (10 mg/ml in 6x NTE) added, incubated at 65°C for 30 min, and allowed to cool to 30 °C. The beads were washed three times in 3x NTE, twice in 1x NTE and once in 0.1x NTE. tRNAs were eluted with 0.1x NTE at 65-68°C, ethanol precipitated and re-dissolved in amino acylation buffer (50 mM Hepes pH 7.5, 25 mM KCl, 15 mM $MgCl_2$, 5 mM DTT). Further purification was achieved using the QIAquick nucleotide removal kit (Qiagen, Hilden, Germany).

The purity of tRNAs was controlled via Northern dot-blot-analysis. 0.5 -1 µg tRNA was crosslinked onto a nylon membrane by UV irradiation and incubated with biotinylated oligonucleotides in 250 mM Na_2HPO_4, 1 mM EDTA, 20 % SDS, 100 mM maleic acid, 150 mM NaCl and blocking reagent (Roche 1096176) overnight at pH 7.5 and 68°C. After 5-fold rinsing in PBS (137 mM NaCl, 2.2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) and 0.1 % Tween 20, streptavidin-AP-conjugate (IBA BioTAGnology, Göttingen, Germany) was added to the membrane and incubated for 90 min at 37 °C, washed 3x in PBS plus 0.1 % Tween 20. The color reaction was performed using nitroblue tetrazolium chloride (NBT) and 5-bromo,4-chloro,3-indolylphosphate (BCIP) [Roth, Karlsruhe, Germany]. Degradation of tRNAs was checked by denaturing 12 % polyacrylamide/8M urea gel electrophoresis.

Aminoacylation assays with GluRS

In vitro acylation experiments with ND-GluRS$_{Tel}$ were carried out at 37°C in a 130 µl reaction mixture containing 50 mM Hepes pH 7.5, 25 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 5 mM ATP, 30 µM L-[^{14}C]Glu (237 mCi/ mmol) and 21 to 0.7 µg of pure tRNA. The reactions were started by addition of the enzyme (1.8 µM GluRS). 15 µl samples were taken at different times, spotted on 3 MM Whatman filter paper discs and washed twice in 10%, once in 5% trichloroacetic acid and finally in ethanol. Uniformly labeled L-[^{14}C] Glu (237 mCi/ mmol) and L-[^{14}C] Gln (210 mCi/ mmol) were purchased from Hartmann Analytic (Braunschweig, Germany).

Crystallization

ND-GluRS$_{Tel}$ was crystallized by hanging drop vapor diffusion at 20°C. 3 µl of protein in 20 mM Hepes pH 7.9, 20 mM NaCl, 10 mM DTT, 250 µM Zn acetate and 250 µM $MgCl_2$ was added to 3 µl of reservoir solution containing 740 mM Na citrate, 140 mM citric acid (final pH 5.8) and 10 mM DTT. Crystals grew to a size of 500 x 100 x 50 µm^3 within 1-2 weeks. Prior to X-ray data collection 25 % (v/v) of a 50 % (w/v) trehalose solution were added for cryo-protection. Crystals belong to space group P2$_1$ with cell constants a = 36.2, b = 99.6 and

c = 182.4 Å. A V_M-value of 2.97 Å^3/Da [35] indicated the presence of two GluRS molecules per asymmetric unit, corresponding to a solvent content of 59%.

Data collection, structure determination and analysis

X-ray diffraction data were collected at the beamline PX I (Swiss Light Source). Data were processed and scaled using the XDS program package[36]. The structure of GluRS from *Thermus thermophilus* (PDB-code 1GLN)[11] was used as a model in molecular replacement using Phaser[37]. CNS was used for rigid body and simulated annealing refinement. REFMAC5 was used for subsequent refinement, including TLS-refinement protocols. Coot was used for manual model building and structural analysis.[38] The structure was validated using WHAT IF and PROCHECK[39]. Molecular depictions were prepared using PyMOL.[40] LSQKAB of the CCP4 program suite[41] was used to calculate root-mean-square deviations. Sequence alignments were performed with ClustalW.

Accession number

The coordinates of the structure have been deposited in the Protein Data Bank (Entry code: 2CFO).

Acknowledgements

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Figure Legends

Scheme 1:

Alternative routes of Gln-tRNA^{Gln} production. Eukaryotes and some bacteria can directly produce Gln-tRNA^{Gln} via GlnRS. Most bacteria express lack GlnRS but possess a non-discriminating GluRS (ND-GluRS) instead. ND-GluRS can charge both tRNA^{Glu} and tRNA^{Gln} with glutamate. The misacylated Glu-tRNA^{Gln} is subsequently amidated to Gln-tRNA^{Gln} by a tRNA-dependent amidotransferase (Glu-AdT or GatCAB), which hydrolyzes glutamine yielding glutamate.

Figure 1:

Structure of non-discriminating GluRS from *Thermosynechococcus elongatus* (ND-GluRS$_{Tel}$). Cartoon representation with transparent surface representation. The two views are rotated by 90°. The catalytic domain is depicted in green, the connective peptide (CP) domain in light brown, the stem contact (SC)-fold domain in yellow, and anticodon-binding domains 1 and 2 in light blue and cyan, respectively. All molecular depictions were produced using PyMOL [40].

Figure 2:

Comparison of ND-GluRS$_{Tel}$ with discriminating GluRS from *Thermus thermophilus* (D-GluRS$_{Tth}$). (A) Sequence alignment with conserved residues shown in black, all others in gray. Rectangles and arrows (colors as in Figure 1) above the alignment indicate α-helices and β-strands, respectively. Red letters highlight the ATP-binding motifs and cyan letters the residues mainly responsible for (non-)discrimination. Insertion and deletions are marked by a Δ. (B) Structural comparison as Cα trace representation. ND-GluRS$_{Tel}$ is colored as in Figure 1, while D-GluRS$_{Tth}$ is depicted in gray. The two views are rotated by 90°. Insertions and deletions are highlighted by ovals.

Figure 3:

Substrate recognition in the active site of ND-GluRS$_{Tel}$. (A) Electron density of the substrate glutamate ($|2F_O\text{-}F_C|$ map contoured at 1σ). Residues involved in binding are shown as sticks. (B) Model of the acceptor end of tRNA^{Glu} of *Thermus thermophilus* and the reaction intermediate glutamyl-AMP in the active site of ND-GluRS$_{Tel}$. The enzyme is shown in surface representation with the catalytic domain in green and the connective-peptide (CP) domain in light brown. tRNA^{Glu} and glutamyl-AMP are depicted as sticks with carbon atoms, respectively, in light orange and green. Oxygen is consistently rendered in red, nitrogen in blue, and phosphorus in purple.

Figure 4:

Structural comparison of the anticodon-binding domains of ND-GluRS$_{Tel}$ (A, C) and D-GluRS$_{Tth}$ (B, D). ND-GluRS$_{Tel}$ is colored as in Figure 1, D-GluRS$_{Tth}$ is colored in gray. The tRNA backbone and the three anticodon nucleotides are depicted in light orange. (A) Surface representation of ND-GluRS$_{Tel}$ with modeled tRNA$^{Glu/Gln}$ and (B) D-GluRS$_{Tth}$ with tRNA^{Glu} (PDB code: 1N77)[2]. (C) Cartoon representation of the anticodon-recognition sites of ND-GluRS$_{Tel}$ and (D) D-GluRS$_{Tth}$. Only residues in contact with the anticodon nucleotides are shown as sticks.

Tables

Table 1: Catalytic constants for Glu-tRNA$^{\text{Glu}}$ and Glu-tRNA$^{\text{Gln}}$ formation by non-discriminating GluRS$_{Tel}$

tRNA	K_m [µM]	k_{cat} [s^{-1}]	k_{cat}/ K_m [s^{-1}mM^{-1}]
Glu-tRNA$^{\text{Glu}}$	0.79	0.1	126
Glu-tRNA$^{\text{Gln}}$	3.7	0.036	9.7

Individual kinetic parameters are based on three independent determinations with standard deviations from 3-10 %.

Table 2: Data collection and structure determination statistics

Data collection	
Unit cell dimensions, a, b, c (Å)	36.2, 99.6, 182.4
Space group	P2$_1$
Wavelength (Å)	0.90
Number of unique reflections	51842
Resolution range (Å)	50-2.45 (2.55-2.45)
Completeness of data (%)	96.9 (91.2)
Redundancy	2.8 (2.4)
R$_{merge}$ (%)	11.2 (42.4)
I/σ	11.2 (3.2)

Refinement statistics	
Maximum resolution (Å)	2.45 (2.51-2.45)
No. of atoms: protein, water	7743, 321
Monomers per asymmetric unit	2
R-factor (%)	18.1 (26.4)
R$_{free}$ (%)	24.8 (32.2)
Average B-factor (Å^2)	17.7

R.m.s.d. bond length (Å)	0.02
R.m.s.d. bond angles (°)	1.50
Ramachandran plot[#]	95.5/4.5/0/0

Values in parentheses refer to shell of highest resolution.
[#] Procheck[39]: most favored/additionally allowed/generously allowed/disallowed region