Deinococcus glutaminyl-tRNA synthetase is a chimer between proteins from an ancient and the modern pathways of aminoacyl-tRNA formation

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ABSTRACT
Glutaminyl-tRNA synthetase from Deinococcus radiodurans possesses a C-terminal extension of 215 residues appending the anticodon-binding domain. This domain constitutes a paralog of the Yqey protein present in various organisms and part of it is present in the C-terminal end of the GatB subunit of GatCAB, a partner of the indirect pathway of Gln-tRNA\textsubscript{Gln} formation. To analyze the peculiarities of the structure–function relationship of this GlnRS related to the Yqey domain, a structure of the protein was solved from crystals diffracting at 2.3 Å and a docking model of the synthetase complexed to tRNAGln constructed. The comparison of the modeled complex with the structure of the E. coli complex reveals that all residues of E. coli GlnRS contacting tRNAGln are conserved in D. radiodurans GlnRS, leaving the functional role of the Yqey domain puzzling. Kinetic investigations and tRNA-binding experiments of full length and Yqey-truncated GlnRSs reveal that the Yqey domain is involved in tRNAGln recognition. They demonstrate that Yqey plays the role of an affinity-enhancer of GlnRS for tRNAGln acting only in \textit{cis}. However, the presence of Yqey in free state in organisms lacking GlnRS, suggests that this domain may exert additional cellular functions.

INTRODUCTION
Flawless protein translation requires a full set of 20 types of perfectly paired aminoacyl-tRNAs. It was de facto expected that each cell should possess a full set of 20 aminoacyl-tRNA synthetases capable of matching each of the 20 natural amino acids to the cognate tRNAs (1). Recent studies on synthesis of glutaminyl-tRNA\textsubscript{Gln} have forced a revision of this assumption (2). This aminoacylated tRNA displays the unique feature of being formed by kingdom-specific pathways (3,4). Eukaryotes and a small subset of bacteria use the common route of tRNA aminoacylation, whereby the GlnRSs attach glutamine directly onto tRNAGln (5). The majority of bacteria and all archaea use an indirect pathway involving a tRNA-dependent amidotransferase that compensates for the absence of GlnRS (3,4,6,7). In this alternate route, glutamyl-tRNA\textsubscript{Gln}, a misacylated species formed by a non-discriminating GluRS (8), is amidated by a domain-specific AdT thereby generating the correctly paired glutaminyl-tRNA\textsubscript{Gln} (3,4). In bacteria, AdTs are composed of three subunits, GatC, GatA and GatB assembled in a heterotrimeric enzyme called GatCAB (4,9,10). While half of the archaea possess this heterotrimeric AdT, they all possess an archaeal-specific AdT called GatDE (3,9–11). Transamidation-catalyzed formation of Gln-tRNA\textsubscript{Gln} is so widespread among prokaryotes that GlnRS is rarely found in bacteria and has so far never been identified in archaea. Deinococcus radiodurans is one of those rare bacteria possessing a GlnRS (12,13).

All aaRSs are assembled piece-wise and often display domains appended to the two ubiquitous functional modules that are the catalytic and anticodon-binding domains (14–18). However, all bacterial GlnRSs studied so far lack any appended domain. The presence in D. radiodurans GlnRS of 220 additional amino acids drew our attention. This extension is located in the C-terminal part of the protein and constitutes an appendix of its anticodon-binding domain (12,19,20). Sequence comparison shows that the C-terminal half of this appendix is homologous to a family of bacterial and yeast proteins of unknown function named Yqey.

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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(PFAM id: PF02637, 21). There are ~100 known Yqey proteins that on average are 150 amino-acid-long, and interestingly, share sequence homologies with the C-terminal domain of GatB and GatE, the tRNA-binding subunits of bacterial and archaean AdTs (22,23). The presence of the gatB or gatE gene signals the presence of an AdT in an organism since the GatB and GatE subunits are exclusively found in association with the proteins that on average are 150 amino-acid-long, and the Yqey protein, have no homologs. Among these proteins, the one encoded by D. radiodurans or D. radiodurans R1 strain was from DSMZ and was grown overnight at 37°C in glass flasks as described (24) and harvested at the end of the exponential phase (A600 nm = 2.5).

MATERIALS AND METHODS

Materials

Hydroxyapatite CHT20 column was from Bio-Rad. L-[14C]Gln (254 mCi·mmol⁻¹) was from Amersham. Dyazyme Taq polymerase from Finnzyme, restriction enzymes, chitin-agarose, pTYB11 vector and E. coli ER2566 strain were from New England Biolabs. D. radiodurans R1 strain was from DSMZ and was grown overnight at 37°C in glass flasks as described (24) and harvested at the end of the exponential phase (A600 nm = 2.5).

Cloning, expression and purification of native and truncated D. radiodurans GlnRs

The ORFs of full length and C-terminal truncated D. radiodurans GlnRs (FL- and Δ-GlnRS) and of full length and N-terminal truncated Yqey domains (FL- and Δ-Yqey) were PCR-amplified using 100 ng genomic DNA and 100 pmol of each of the following primers: 5'-GGTGGG TGCTTCACACATGGGGGCGTTGGTGG C-3' (sense for FL- and Δ-GlnRS) and 5'-GGTG GTCTGCAGTCATTAGGCGAGAGCGTCTTTAGG CCGC-3' (anti-sense for FL-GlnRS) and 5'-GGTG TCGTCGTCATAGGCGGACAGGTCTTTAGG GC-3' (anti-sense for FL-Yqey) and 5'-GGTGTTGGT CAGTCATTAGGCCCGCTGCTGCCCCAGG-3' (anti-sense for Δ-GlnRS), 5'-GGTGTTGGTCGTCATTAGGCGACAGCGTCTTTAGG CCGC-3' (sense for Δ-GlnRS) and FL-GlnRS purification were conducted as described (24). Δ-GlnRS, FL- and Δ-Yqey domains were purified on chitin-agarose and elution by self-cleavage of intein followed by chromatography on Bio-Scale CHT5-I ceramic hydroxypatite. Purity was controlled by SDS-PAGE and mass spectrometry. Proteins were stored at −20°C in 50 mM Tris-HCl pH 8.0 containing 5 mM β-mercaptoethanol, 0.5 mM Na2-EDTA and 50% glycerol. Molecular mass of Dr GlnRS was determined by mass spectrometry on a Bruker Biflex III apparatus.

Total tRNA and tRNAGln transcripts from D. radiodurans

Bacterial pellets (80 g) were treated with 95% ethanol to remove the cell outer membrane. After centrifugation, cells were suspended in 140 mM Na-acetate pH 4.5 containing 0.1% SDS. Nucleic acids were extracted with acid-buffered phenol and precipitated with ethanol. RNAs were recovered by centrifugation and total RNA was obtained by isopropanol precipitation and chromatography on a DEAE-cellulose column (25). Deinococcus radiodurans tRNA(Gln(UUG)), and tRNA(Gln(CUC)) transcripts were obtained by in vitro transcription of the genes cloned under control of the T7 RNA polymerase promoter using the cassette cloning procedure (26). Since the two tRNAs start with 5'U known to prevent in vitro transcription, both genes were preceded by a sequence encoding a hammerhead ribozyme. Ribozyme-catalyzed self-cleavage and purification of transcripts were conducted as described (27).

Protein immunodetection

Sera containing antibodies directed against Dr FL-GlnRS (anti-FL-GlnRS) or Thermus thermophilus GatCB AdT (anti-AdT) were obtained from immunized rabbits (28). Western-blotting was performed with 0.05–1 μg of purified proteins electrophoresed onto a nitrocellulose membrane (PROTRAN BA 83, pore size 0.2 μm, Schleicher and Schuell) after 10% (for FL-Yqey and Δ-Yqey) SDS-PAGE. Membranes were incubated 1.5 h at room temperature in 50 μl of 20 mM Tris-HCl pH 7.5 containing 500 mM NaCl, 0.05% Tween and anti-FL-GlnRS or anti-AdT sera diluted 2000-fold. Antibodies bound to proteins were detected with peroxidase-conjugated anti-rabbit IgG in the presence of a bioluminescent reagent (ECL Kit, Amersham Bioscience). The ternary complexes were revealed by exposition of the membrane for 2–15 s on a X-ray film.

Gel-shift assays

Deinococcus radiodurans tRNA(Gln(UUG)) transcript was [32P]-labeled at the 3′-end by exchange of its CCA terminus. Five pmoles of transcript in 50-μl reaction mixture containing 50 mM Tris-HCl pH 8.0 and 10 mM MgCl2 were treated with 10⁻³ unit of Crotalus venom phosphodiesterase for 10 min at 20°C. After phenol extraction and ethanol precipitation, the transcript without CCA, was incubated 30 min at 37°C in 50-μl reaction mixtures containing 500 ng tRNA and 500 ng of [32P]-labeled tRNA.
mixture containing 1.8 μg of *E. coli* trinucleotidyl-tRNA transferase, 50 μCi α-[32P]ATP, 50 μM CTP, 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂ and 8 mM DTE. The radiolabeled tRNA was purified by 8 M urea PAGE (10%), electroeluted, precipitated with ethanol and renatured.

Complexes between GlnRS and Yqey and their variants and tRNA<sub>Gl</sub> were visualized by band-shift after PAGE fractionation and quantified. For that, constant amounts of (tRNA<sup>Gln</sup>) transcript (5 × 10<sup>5</sup> cpm) were incubated with increasing amounts of FL-GlnRS, Δ-GlnRS, FL-Yqey or Δ-Yqey in the presence of 10% glycerol, 12 mM MgCl₂ and 30 mM KCl during 20 min at room temperature. Some assays contained 50 nM of *Neisseria meningitidis* and 30 mM KCl during 20 min at room temperature.

Activity assays
ATP-[32P]PPi exchange was performed in 300 μl reaction mixtures containing 100 mM Na-HEPES pH 7.2, 5 mM MgCl₂, 2 mM ATP, 2 mM [32P]PPi (1–2 cpm pmol⁻¹), 1 mM Gln, 0.5 mg ml⁻¹ bovine serum albumin, 2 mg ml⁻¹ total D. radiodurans tRNA and appropriate amounts of FL- or Δ-GlnRSs. For K<sub>M</sub> measurements, concentrations of tRNA<sup>Gln</sup> and Gln varied, respectively, from 0.1 to 3 μM and from 0.7 to 10 mM for FL-GlnRS and from 0.5 to 10 μM and from 1 to 20 mM for Δ-GlnRS, the non-varied ligands being saturating. The [32P]ATP formed at 37°C was determined in 50-μl aliquots (29).

For tRNA aminoacylation, 25 μl reaction mixtures containing 100 mM Na-HEPES pH 7.2, 2 mM ATP, 10 mM MgCl₂, 30 mM KCl, 0.5 mg ml⁻¹ bovine serum albumin, 50 μM L-[¹⁴C]Gln (351 cpm pmol⁻¹), 1–2 mg ml⁻¹ D. radiodurans tRNA and appropriate amounts of FL- or Δ-GlnRSs. For K<sub>M</sub> measurements, concentrations of tRNA<sup>Gln</sup> and Gln varied, respectively, from 0.1 to 3 μM and from 0.7 to 10 mM for FL-GlnRS and from 0.5 to 10 μM and from 1 to 20 mM for Δ-GlnRS, the non-varied ligands being saturating. The [32P]ATP formed at 37°C was determined in 50-μl aliquots (29).

Crystallographic methods, structure analysis and modeling
Crystals of Dr GlnRS diffracting at 2.3 Å resolution were grown in microbatch in the presence of PEG 3350 (24). The structure was solved by molecular replacement (MR) using the structure of Ec GlnRS as a search model (30). The MR solution was refined with CNS (31) using a maximum likelihood target, a bulk solvent correction and a set of 7% of the reflections randomly selected for free R-factor testing. After rigid-body refinement, alternate simulated annealing and B-factor refinement rounds followed by a stepwise increase of resolution from 3 to 2.3 Å, the R- and free R-factors dropped from 50.9 and 51.1% to 25.6 and 29.8%, respectively. Several loops of the model were rebuilt manually in O (32). Water molecules developing sensible H-bonds with protein or other solvent atoms were finally added, leading to final R- and free R-factors of 20.0 and 24.2%, respectively. The final model includes 556 amino acids (A43–W632) and 160 water molecules. The N-terminal tail (M1–P42), the C-terminal extension (G633–A852) and two loops in the anticodon-binding domain (N537–Q564 and D614–A619) are not seen even in electron density maps computed at lower resolutions. Structure validation was performed with PROCHECK (33). The structure has been deposited with the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB code: 2HZ7).

Structures of Ec GlnRS, either in the free state (PDB id: 1NYL) or in complex with ligands (tRNA, AMP, Gln; PDB id: 100B) were used for comparison with that of Dr GlnRS using LSQM (34). Superimposition was performed with a distance cutoff of 3.5 Å. Root-mean-square deviation (RMSD) values determined for the whole GlnRS-core or individual domains are based on equivalent main chain positions (N, Ca, C). This structural superimposition allowed annotation of the alignment.

A normal mode analysis of the structure of Dr GlnRS based on the elastic network model was performed using the EL-Nemo web service (34,35). Sets of normal mode perturbed models were generated for the first five low frequency modes. The model showing the lowest RMSD with Ec GlnRS complexed with the ligands obtained by combining mode 7 and 9 was used for tRNA docking. A series of models, intermediate between the latter and the X-ray structure, were used to predict a plausible conformational pathway between the free and tRNA-bound states. The regions of the enzyme that are not observed in the electron density map and, thus are absent in the current structure, were modeled using Modeller (36). The C-terminal domain was derived from alignment of the Yqey domains and the structure of the Yqey protein of *Bacillus subtilis* (PDB id: 1NG6).

RESULTS

*Dr GlnRS* is a structural hybrid between conventional GlnRS and AdT

The structural organization of Dr GlnRS was established by separate multiple sequence alignments. A first alignment of known bacterial GlnRSs shows that Dr GlnRS is highly similar to other bacterial GlnRSs, with the exception of a few insertions (17, 27 and 5 additional residues in the structured region 410–433 and in two loops 535–565 and 610–620, respectively) and the presence of a short N-terminal extension (M1–A43) and a long C-terminal appendix (G633–A862) (Figure 1A). This high degree of homology suggests that Dr GlnRS,
like the *E. coli* enzyme (30), has a modular architecture (Figure 1B). Moreover, most residues contacting substrates in *Ec* GlnRS (20,30) are conserved in *Dr* GlnRS.

Next, the C-terminal appendix of *Dr* GlnRS was aligned with all known Yqey sequences, including that of *B. subtilis*, and with those of bacterial GatB and GatE proteins (Figure 1C). This alignment shows a moderate degree of sequence similarity, since out of the 215 amino acids constituting the extension of *Dr* GlnRS, only the 150 most distal align with other Yqey proteins, with highest similarity in the 30 last residues (Figure 1C).

The relatively weak sequence homology between the Yqey domain of *Dr* GlnRS and GatB subunits raises the question of their conformational homology. A first answer came from an immunological approach. Western blots (Figure 2A) show that *Dr* GlnRS is recognized by anti-AdT antibodies (lane 2) specific to the GatA and GatB subunits of *T. thermophilus* AdT (lane 1). These antibodies do not recognize *T. thermophilus* GlnRS (Figure 2A, lane 3) lacking Yqey (39), suggesting that anti-GatB antibodies recognize the Yqey domain of *Dr* GlnRS. The unambiguous proof of this assumption came from comparative experiments done with three GlnRS variants, namely a truncated form lacking the entire Yqey domain (*C1*-GlnRS), a full length Yqey domain of 220 amino acids (FL-Yqey) and a deletion variant of the extension without the 61 residue-long linker (*C1*-Yqey).

/C1*-GlnRS is no longer recognized by anti-AdT antibodies.
the anti-GatB antibodies, while both FL-Yqey and Δ-Yqey are recognized (Figure 2A, lanes 5–7). Thus, the Yqey domain of DrGlnRS is solely responsible for the cross-reaction with anti-GatB antibodies. Conversely, antibodies directed against DrGlnRS, specifically recognize GatB from *T. thermophilus* AdT and not GatA (Figure 2A, lanes 8 and 9). Additionally, these antibodies are unable to recognize *T. thermophilus* GlnRS indicating the absence of common epitopes in the core of both enzymes (Figure 2A, lane 10). Altogether this demonstrates a structural resemblance between the Yqey domain of DrGlnRS and the family of Yqey proteins. In other words, it confirms the chimerical nature of DrGlnRS suspected from sequence analysis.

### Crystallographic structure of DrGlnRS

FL-GlnRS forms orthorhombic crystals that diffract X-rays beyond 2.3 Å resolution. The structure was solved by molecular replacement with the *E. coli* enzyme in complex with the ligands (PDB id: 1O0B) giving the best score (40). In total, 556 residues corresponding to the classical GlnRS-core in the N-terminal region of the protein were built and refined. Unexpectedly, about one-third of the protein is not observed in the crystal packing, despite the good quality of the diffraction data (Table 1) and the resulting electron density maps (Supplemental Figure 1). The missing parts are the N-terminal tail, two loops of the GlnRS-core and the C-terminal domain. A proteolytic degradation is excluded since the integrity of the protein in the crystals was confirmed by SDS-PAGE and mass spectrometry. This suggests that the missing parts of the protein are not trapped but remain mobile to a certain extent in the packing, an interpretation supported by additional physico-chemical investigations (to be published). At the present stage, analysis of crystal packing and solvent content (44% and Matthews coefficient 2.2 Å³/Da) indicates that the P2₁2₁2₁ crystals can accommodate FL-GlnRS. This implies that the ordered part of the protein only represents 38% of the unit cell volume.

As could be anticipated from sequence conservation and straightforward molecular replacement, the GlnRS-core is similar to that of *E. coli* (Figure 3A). The structures can be superimposed with a RMSD of 1.9–2.3 Å (Supplemental Table 1 for RMSD analysis). Below, the *E. coli* nomenclature is used to describe the protein which can be divided as follows: an active site domain (ASD) composed of a Rossmann or dinucleotide fold (DNF) and the acceptor-stem binding domain (ABD), an intermediate helical subdomain (HSD) and the anticodon-binding domain (ACD) made of a proximal and a distal β-barrel (PBB, DBB).
Follows the nomenclature of the Domains of the latter are depicted in distinct colors and their definition in gold) is superimposed to the visible core of Dr GlnRS with cognate tRNA, Gln and AMPcPP (40) (overall structure colored beta-barrels in violet and magenta, respectively. (A) Domain in light blue, helical subdomain in green, proximal and distal to C-terminus: dinucleotide fold in dark blue, acceptor-stem binding superimposed with a distance lower than 3.5 Å. In contrast, white GlnRS highlights in orange and red all common positions that are conserved between Ec GlnRSs is deprived of any insertion–deletion sequence. This part of both the two proteins are similar and present the HI/LGH and T/MSK signatures of class I synthetases. This part of both GlnRSs occurs in their ACDs. The PBB of Dr GlnRS whereas Ec GlnRS: all residues involved in recognition of Gln by Dr GlnRS shown to participate in binding of the substrates are almost fully conserved in Dr GlnRSs occur in their ACDs. The PBB of Dr GlnRS whereas Ec GlnRS whereas Dr GlnRS contacting ATP, 8 are conserved or substituted by equivalent residues and the 13 contacting Gln are conserved, except one substituted by a functionally equivalent residue (M of the MSK motif replaced by T) (Supplemental Table 2).

Figure 3 highlights the sequence and structure conservations between Ec and Dr GlnRSs. The ASDs of the two proteins are similar and present the HI/LGH and T/MSK signatures of class I synthetases. This part of both GlnRSs is deprived of any insertion–deletion sequence. The unique difference concerns position of a helix in the ABD which varies from more than 3.5 Å (depicted in white in Figure 3B). It adopts an open conformation in Dr GlnRS and closes upon tRNA binding in the ligand-complexed Ec GlnRS. Overall, low RMSD values (0.8–1.3 Å) are observed in the ASD, as well as in the HSD.

The major structural differences between Ec and Dr GlnRSs occur in their ACDs. The PBB of Dr GlnRS contains two insertions in surface loops that are not seen in the density. The first consists of 27 residues forming an extended loop (535–565) located at the bottom of the catalytic cleft; this element is replaced by a short turn in Ec GlnRS. The second concerns five additional residues in a loop (610–620) opposite to the anticodon-loop binding site. The PBB has been proposed as a module promoting communication between the catalytic center and the DBB where the anticodon of tRNA is bound. Accordingly, this part of the structure shows the highest RMSD deviations with the complexed Ec GlnRS. The DBB of Dr GlnRS also carries other idiosyncrasies, namely an additional element of 34 residues (410–433) that folds in a helix followed by two short β-strands sitting next to the minor groove of the anticodon stem of tRNA and a deletion of 8 residues found in a loop that would be close to nucleotides 34–36 of the tRNA anticodon. This loop (497–505) is well defined in Ec GlnRS whereas it has not been observed in the structure of Ec GlnRS either in free form or complexed with the substrates, probably due to a greater mobility related to its larger size. Overall, this domain appears to be more rigid than the PBB as revealed by RMSD values of 1.2–1.3 Å.

The amino acids of Ec GlnRS shown to participate in binding of the substrates are almost fully conserved in Dr GlnRS: all residues involved in recognition of Gln by direct or solvent mediated interactions are present, as well as most residues constituting the ATP pocket. Even more striking is the high level of conservation of the residues which in Ec GlnRS develop specific interactions with tRNA (Figure 1A).

Among the 40 residues of Ec GlnRS which contact tRNA (12 with main-chain atoms and 28 with lateral-chain atoms) 25 are strictly conserved and 9 present structural conservative changes (e.g. Ile/Leu, Glu/Gln) in Dr GlnRS. Interestingly, 4 residues of Dr GlnRS are unable to make the interactions described for the E. coli complex. Among the 10 residues of Ec GlnRS contacting ATP, 8 are conserved or substituted by equivalent residues and the 13 contacting Gln are conserved, except one substituted by a functionally equivalent residue (M of the MSK motif replaced by T) (Supplemental Table 2).

Effect of Yqey on the kinetic properties of GlnRS

Preliminary assays with total D. radiodurans tRNA showed same aminoacylation plateaus with Δ- and FL-GlnRSs, suggesting that Δ-GlnRS does not mischarge a non-cognate tRNA. The plateau obtained with FL-GlnRS does not increase after addition of Δ-GlnRS, showing that both enzymes charge the same tRNA. However, to obtain the same plateau, a significantly higher concentration of Δ-GlnRS than of FL-GlnRS was necessary.

Table 1. Summary of X-ray data collection and structure refinement statistics

| Data collection | X-ray source | ID14-1 (ESRF) |
|-----------------|--------------|---------------|
| Space group     | P2₁2₁2₁      |               |
| Cell parameters | a, b, c (Å)  | 74.1, 95.9, 115.7 |
| Resolution (Å)  | 2.3–30       |               |
| No. of observations | 209 589 | |
| No. of unique reflections | 36 991 | |
| Completeness (%) | 98.1 (92.9)³ | |
| Rmerge (%)      | 6.2 (25.0)³ | |
| Average I/σ(I)  | 18.1 (5.5)³ | |
| Refinement      |              |               |
| R-factor (%)    | 20.0 (25.5)³ | |
| Free R-factor (%)| 24.2 (29.1)³ | |
| No. of atoms    | Protein      | 4488          |
|                 | Water molecules | 160         |
| Average B-factors (Å²) | Overall | 41.7        |
|                 | Protein      | 41.6          |
|                 | Water molecules | 42.3        |
| RMSD for bonds (Å), angles (°) | 0.009, 1.5 | |
| Ramachandran plot (%) | Most favored | 90.2       |
|                 | Additional allowed | 9.3         |
|                 | Generously allowed | 0.4         |

³Last resolution shell: 2.30–2.36 Å.
and C1/C1

Table 2. Activities of full length and C-terminal truncated D. radiodurans GlnRS

| Substrate | ATP-[^32]PPi, exchange | tRNA Aminoacylation |
|-----------|------------------------|---------------------|
|           | FL-GlnRS | Δ-GlnRS | KM (μM) | FL-GlnRS | Δ-GlnRS | KM (μM) |
| Gln       | 1000 | 2130 | 2.13 | 186 | 168 | 0.9 |
| tRNA_{Gln} (n) | 0.062 | 6.25 | 101 | 0.064 | 1.54 | 24 |
| tRNA_{Gln} (t) | nd | nd | nd | 0.120 | >27 | >225 |
| tRNA_{Gln} (n) | k_{cat} (s^{-1}) | Lk_{cat} | 5.73 | k_{cat} (s^{-1}) | Lk_{cat} | 4.6 |
| tRNA_{Gln} (t) | 44 | 26 | 1.7 | 0.15 | nm | nm |
| tRNA_{Gln} (n) | 710 | 4.2 | 170 | 89.5 | 0.83 | 1.25 |
| tRNA_{Gln} (t) | nd | nd | nd | 4.5 x 10^{-6} | 2.78 x 10^{3} |

Each value is an average of at least two independent determinations. tRNA_{Gln} (n): native modified tRNA_{Gln} in total D. radiodurans tRNA; tRNA_{Gln} (t): tRNA_{Gln(UUG)} transcript. Losses in affinity for the ligands (Lk_{M}), in the catalytic rate (Lk_{cat}) and in catalytic efficiency (Lk_{cat}/Lk_{M}) between FL- and Δ-GlnRSs are expressed as K_{M} of Δ-GlnRS over K_{M} of FL-GlnRS, k_{cat} of FL-GlnRS over k_{cat} of Δ-GlnRS and k_{cat}/K_{M} of FL-GlnRS over k_{cat}/K_{M} of Δ-GlnRS, respectively. nd: not determined; nm: non measurable. Standard deviation of data is ~20%.

is required, indicating that deletion of Yqey alters the catalytic properties of the enzyme.

Comparative experiments deciphered the effects of Yqey on the catalytic properties of Dr GlnRS. First Δ-GlnRS, like FL-GlnRS and other GlnRSs, requires cognate tRNA for Gln activation. Further, the kinetic constants of Gln activation and tRNA aminoacylation (Table 2) show that both FL- and Δ-GlnRSs exhibit similar K_{M}s for Gln in ATP–PPi exchange as well as in tRNA charging, suggesting that Yqey is not involved in binding of the amino-acid substrate. This activation is specific since the two enzyme forms do not catalyze Glu-dependent ATP–[^32]PPi exchange under the conditions where Gln promotes the exchange and despite the ability of Ec GlnRS to bind Glu (40). On the other hand, FL- and Δ-GlnRSs catalyze amino-acid activation and tRNA aminoacylation, with similar rate since deletion of the Yqey domain only slightly decreases k_{cat}'s of ATP–PPi exchange and tRNA charging (Table 2). This suggests that Yqey does not contain essential residues for catalysis or active conformation of the enzyme. The domain, however, increases the affinity of the enzyme for cognate tRNA_{Gln}, as revealed by the significant K_{M} decrease of FL-GlnRS for this substrate, especially pronounced for ATP–PPi exchange. Taken together, the Yqey flexible module increases the catalytic efficiency of GlnRS for both tRNA_{Gln} dependent Gln activation and tRNA_{Gln} charging and is involved in binding of tRNA_{Gln} to GlnRS.

Next, the capacity of Dr GlnRS to make a functional distinction between its two tRNA_{Gln} isoacceptors, tRNA_{Gln(UUG)} and tRNA_{Gln(CUG)}, and the possible role of Yqey in this distinction, were investigated. Thus charging of the two transcripts by FL- and Δ-GlnRSs was tested. In contrast to Ec GlnRS that charges transcript and modified tRNA_{Gln} with similar efficiencies (42), Dr GlnRS aminoacylates the tRNA_{Gln(UUG)} transcript significantly less efficiently than modified tRNA_{Gln} and the two transcripts with distinct rates, the tRNA_{Gln(UUG)} transcript being charged one order of magnitude faster than the tRNA_{Gln(CUG)} transcript (not shown). Further, Δ-GlnRS charges the tRNA_{Gln(UUG)} transcript one order of magnitude slower than FL-GlnRS and is unable to charge the tRNA_{Gln(CUG)} transcript to a detectable level (not shown). This indicates that Dr GlnRS can charge the two isoacceptors, although with distinct efficiencies and that Yqey does not support preferential aminoacylation of one of them. Interestingly, the two tRNAs display 73% identity. Sequence analysis shows that among the elements determining glutaminylation of E. coli tRNA_{Gln} (43–45), 10 are conserved in D. radiodurans tRNA_{Gln(UUG)} (G73, G2–C71, G3–C70, G10, U34, U35 and G36, U38) and 8 in tRNA_{Gln(CUG)} (G73, G2–C71, G3–C70, G10, U35, G36, U1–A73). Among the positions involved in E. coli glutaminylation, both tRNAs differ by positions 1–73, 34 and 38 (respectively A1–U73, U34, U38 and U1–A73, C34, C38 in tRNA_{Gln(UUG)} and tRNA_{Gln(CUG)}).

Interestingly, the above data suggest a role of post-transcriptional modifications in tRNA aminoacylation (Table 2). FL-GlnRS aminoacylates modified tRNA_{Gln(UUG)} ~80-fold more efficiently than the transcript as a result of a strong k_{cat} increase and a faint K_{M} decrease. The poor affinity of Δ-GlnRS for the tRNA_{Gln(UUG)} transcript did not allow determination of the individual kinetic constants for the unmodified molecule. However, under first-order kinetic conditions with respect to tRNA, the k_{cat}/K_{M} ratio could be measured directly. It indicates that Δ-GlnRS aminoacylates the transcript ~5 order of magnitude less efficiently than FL-GlnRS. Since the absence of Yqey decreases charging efficiency of the transcript much more than that of native tRNA_{Gln} (~23 000-fold), participation of the post-transcriptional modifications in the Yqey-dependent formation of the competent GlnRS-tRNA_{Gln} complex is not a side effect. It is specific to D. radiodurans tRNA_{Gln} since modified E. coli tRNA_{Gln} is a poor substrate for Dr GlnRS (not shown). The nature of the peculiar modifications in D. radiodurans tRNA_{Gln} awaits
to be characterized. Interestingly, it has been shown that in E. coli the post-transcriptionally modified nucleotides of tRNA Gln are not involved in glutaminylation (44).

Effect of Yqey on tRNA binding on GlnRS

Binding of tRNA\textsuperscript{Gln} on FL- and Δ-GlnRSs, and on FL-Yqey, was analyzed by gel retardation. Figure 4A shows, as expected, a shift of the \textsuperscript{32}P-labeled tRNA\textsuperscript{Gln(UUG)} transcript when migrating with FL-GlnRS. An apparent dissociation constant (\(K_D\)) of 2.8 \(\mu\)M could be calculated for the complex (Figure 4D). Binding is specific as incubation with a 100-fold excess of tRNA\textsuperscript{Asn} transcript did not displace tRNA\textsuperscript{Gln(UUG)} from the enzyme. In contrast, Δ-GlnRS is incapable to interact with tRNA\textsuperscript{Gln(UUG)} transcript throughout the range of protein concentrations assayed, as no mobility shift of labeled tRNA occurs (Figure 4B). Further, no shift was observed when increasing Δ-GlnRS concentration up to 50 \(\mu\)M. This confirms that removal of Yqey suppresses the tRNA-binding capacity of Δ-GlnRS.

Given this crucial contribution of Yqey in Δ-GlnRS for binding tRNA\textsuperscript{Gln}, the capacity of FL-Yqey (Figure 4C) and Δ-Yqey domains to bind the tRNA\textsuperscript{Gln(UUG)} transcript was checked. Both Yqey variants are unable to shift the cognate tRNA transcript, even at very high protein concentrations. Thus, isolated Yqey is not a tRNA binder \textit{per se}. Furthermore this domain is unable to restore in \textit{trans} (i.e. by addition of increasing amounts of FL- or Δ-Yqey domains) a tRNA\textsuperscript{Gln}-binding capability to Δ-GlnRS (Figure 4C).

The GlnRS-tRNA\textsuperscript{Gln} complex of D. radiodurans

To gain insight into the implication of each region of the Δ-GlnRS in substrate binding, a model of the FL-GlnRS in complex with tRNA\textsuperscript{Gln} was built (Figure 5). The Δ-GlnRS was docked onto the Ec GlnRS-tRNA\textsuperscript{Gln} complex and the missing C-terminal domain was modeled using the structure of B. subtilis Yqey protein. The Yqey extension could be easily constructed in the architecture found in the crystal structure of B. subtilis Yqey (PDB id: 1NG6). Its location in Figure 5 is revealing since it can move on the flexible hinge linker, as implied by its disordered location in the Δ-GlnRS crystals that otherwise show good perfection. The model indicates that the Yqey domain can easily interact with any region of the GlnRS core or of the tRNA. In addition, normal mode analysis suggests a conformational pathway similar to that described for the Ec GlnRS-tRNA\textsuperscript{Gln} complex: a closure of the enzyme upon tRNA binding and a simultaneous rotation of the anticodon-binding domain with respect to the catalytic core (not shown).

DISCUSSION

Unprecedented properties of the Yqey domain from Δ-GlnRS

As the EMAPII-like domain present in aaRSs and involved in tRNA binding, Yqey exists either in a free form or as an aaRS-appendix. However, contrasting with free EMAPII formed through a proteolytic cleavage of the p43 protein (46) and to which a cellular function...
could be attributed (47–49), Yqey is synthesized in free form in many bacteria like *B. subtilis* (21), and its function, remains elusive. In yeast mitochondria, where it constitutes the Pet-112 protein, it can be complemented by *B. subtilis* GadB, a paralog of Yqey (50). Notably, Yqey is found only exceptionally in aaRSs and has been identified in only 3 GlnRSs, namely those of *Deinococcus geothermalis* and *Salinibacter ruber* (Blast NCBI) in addition to that of *D. radiodurans* (this study). It constitutes the unique additional domain found in prokaryotic GlnRSs.

The present study establishes that the Yqey domain of *Dr* GlnRS is an actor in tRNA aminoacylation and suggests that it contributes to tRNA binding. More precisely, kinetic data unambiguously show that Yqey is a tRNA affinity enhancer for *Dr* GlnRS. Yqey must be covalently attached to the GlnRS core to fulfill its role, since it is unable to act in *trans* when added in free form to the truncated enzyme. Its connection through a long polypeptide linker to GlnRS constitutes another peculiarity distinguishing Yqey from other aaRS-appended modules. This feature confers unprecedented properties to *Dr* GlnRS.

Formation of the catalytically competent GlnRS-tRNA\textsuperscript{Gln} complex triggered by Yqey is tuned by the modified nucleotides of tRNA, as supported by kinetic data. Although the details of this tuning remain to be deciphered, it appears that the modifications contribute more to the formation of a competent complex than to the primary binding of tRNA. The way whereby Yqey enhances affinity of *Dr* GlnRS for native post-transcriptionally modified tRNA\textsuperscript{Gln} is intriguing since the core enzyme conserves most of the residues of *Ec* GlnRS involved in tRNA recognition, and thus is expected to be fully competent to charge efficiently tRNA\textsuperscript{Gln}. Because appended Yqey increases $k_{cat}$ of tRNA charging and decreases $K_d$, it may act by two ways, either in favoring formation of the competent complex by distorting and reorienting the partners to a productive state or in increasing affinity of GlnRS for tRNA\textsuperscript{Gln} by enhancing the rate of complex formation and decreasing that of its dissociation.

Finally, from the viewpoint of prokaryote genomes, Yqey genes are often located in an operon (Supplemental Table 3). The gene of ribosomal S21 protein is mostly found associated with that of Yqey, but other genes can also be found, although less frequently. The grouping of Yqey and S21 genes in an operon is independent of the presence or absence of AdT and when present, independent of its activity, either Glu- or Asp-AdT. Protein S21 is peculiar to eubacteria and S21-deficient 30S ribosomes are unable to bind mRNA and to initiate protein synthesis in *E. coli* (51). Assuming that Yqey-containing operons encode proteins of related functions, Yqey may be a cofactor involved in protein synthesis.

**Expected features and idiosyncrasies in atypical *Dr* GlnRS: functional implications**

Sequence comparison of *Dr* GlnRS with other bacterial GlnRSs predicts architectural similarities of these enzymes. The prediction was verified by crystallography, with the core of *Dr* GlnRS similar to that of *Ec* GlnRS. Given this fact, and because most of the residues involved in substrate binding are conserved, one could a priori expect that *Dr* GlnRS would be fully functional in the absence of its Yqey appended domain. This is not the case and the serendipitous findings of a Yqey participation in tRNA binding and of the poor binding capacity of Δ-GlnRS, were surprises. Moreover, the Yqey domain by itself is a very weak tRNA binder. These surprising observations can be rationalized assuming the Yqey extension is involved in the formation of the productive GlnRS-tRNA\textsuperscript{Gln} complex.

In a structural perspective, and at first glance, the insertions observed in the core enzyme cannot easily account for the observations. However, although crystallography does not yet answer unambiguously how the extension participates in the aminoacylation function of *Dr* GlnRS, it does not exclude a direct contact of the Yqey domain with tRNA. This domain contains helical subdomains that may allow such contacts with tRNA helices, as found in the yeast AspRS extension (52), but their content of basic residues is poor, except for the most proximal helix to the GlnRS core (Figure 1). The linker making the connection with this core is long enough to allow Yqey to contact any region of the tRNA, even its acceptor or anticodon ends (Figure 5).

If so, the extension would clamp the tRNA on the GlnRS core in position optimal for activity. Reminiscent to what occurs in the *Ec* GlnRS-tRNA\textsuperscript{Gln} complex, where base-pair U1–A72 opens prior entry of the tRNA\textsuperscript{Gln}-acceptor end in the catalytic site (30), and in AdT-systems where this pair is a recognition determinant for amidation of tRNA-bound Glu or Asp into Gln or Asn (23,53), Yqey in *Dr* GlnRS may trigger the formation of the competent complex by contacting the U1–A72 pair of tRNA\textsuperscript{Gln}. Additional structural data are awaited to test this hypothesis and to decipher the puzzling tRNA recognition by *Dr* GlnRS. However, the presence of Yqey in free form regardless whether GlnRS is present or absent in the organism such as in *B. subtilis* deprived of GlnRS and which uses the indirect pathway for Gln-tRNA\textsuperscript{Gln} formation (4), suggests that this domain may exert additional functions not directly related to Gln-tRNA\textsuperscript{Gln} formation, like stabilization of the aa-tRNA, regulation of its synthesis, or coupling of its synthesis with other metabolic pathways. Alternatively, it cannot be excluded that even in free form Yqey participates in cellular functions involving protein–nucleic acids interactions such as binding to an aaRS-tRNA complex to increase efficiency and/or specificity of tRNA aminoacylation. In this case, the protein’s properties would resemble Ybak (54,55) which binds Cys-tRNA\textsuperscript{Pro} complexed with ProRS and promotes hydrolysis of the mischarged tRNA. However, it does so by acting exclusively in *trans*, in contrast to the role of Yqey in *Dr* GlnRS, which can be performed only when it is present in *cis*.

Considerations about evolution of tRNA glutaminylation and asparaginylation led to the proposal that the transamidation pathway constitutes the primitive route to Gln-tRNA\textsuperscript{Gln} and Asn-tRNA\textsuperscript{Asn} formation (9,10).
These routes preceded the emergence of the modern GlnRS and AsnRS, able to synthesize their corresponding aa-tRNAs by charging preferred Gln and Asn onto their cognate tRNAs (9,10). Thus, by amidating Glu and Asp charged on tRNAGlu and tRNAGln, ADT was probably the first enzyme able to form Asn and Gln for protein synthesis purposes. Sequence comparisons and phylogenetic trees revealed that GlnRS evolved from a duplicated gene of GluRS in eukaryotes, and was transferred in various eu-bacterial species by lateral gene transfer (5). In D. radiodurans, Gln-tRNA\textsubscript{Gln} is formed by the modern GlnRS whereas Asn-tRNA\textsubscript{Asn} is formed by the indirect pathway involving ADT (13). In this organism, by GlnRS, of a domain belonging to the ADT, a partner of the ancient pathway of Gln-tRNA\textsubscript{Gln} and Asn-tRNA\textsubscript{Asn} synthesis is surprising, and reveals an unexpected interrelation between the ancient and the modern pathways of amide aa-tRNA formation. Since the Yqey domain is only exceptionally appended to bacterial GlnRS, fusion of both occurred late in evolution and was probably related to acquisition of peculiar properties of GlnRS. Investigations are underway to identify the precise functions of the free and GlnRS-bound Yqey.

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