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Rescuing an essential enzyme–RNA complex with a non-essential appended domain

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Certain protein–RNA complexes, such as synthetase–tRNA complexes, are essential for cell survival. These complexes are formed with a precise molecular fit along the interface of the reacting partners, and mutational analyses have shown that amino acid or nucleotide substitutions at the interface can be used to disrupt functional or repair non-functional complexes. In contrast, we demonstrate here a feature of a eukaryote system that rescues a disrupted complex without directly re-engineering the interface. The monomeric yeast *Saccharomyces cerevisiae* glutaminyl-tRNA synthetase, like several other class I eukaryote tRNA synthetases, has an active-site-containing 'body' that is closely homologous to its *Escherichia coli* relative, but is tagged at its N-terminus with a novel and dispensable appended domain whose role has been obscure. Because of differences between the yeast and *E.coli* glutamine tRNAs that presumably perturb the enzyme–tRNA interface, *E.coli* glutaminyl-tRNA synthetase does not charge yeast tRNA. However, linking the novel appended domain of the yeast to the *E.coli* enzyme enabled the *E.coli* protein to function as a yeast enzyme, *in vitro* and *in vivo*. The appended domain appears to contribute an RNA interaction that compensates for weak or poor complex formation. In eukaryotes, extra appended domains occur frequently in these proteins. These domains may be essential when there are conditions that would otherwise weaken or disrupt formation of a critical RNA–protein complex. They may also be adapted for other, specialized RNA-related functions in specific instances.

Keywords: aminoacylation/*E.coli*–yeast hybrid protein/RNA interactions/tRNA recognition

Introduction

Class I tRNA synthetases typically are comprised of two major domains roughly divided between the N- and C-terminal halves of the respective proteins. The N-terminal domain is made up of alternating β-strands and α-helices arranged in a nucleotide binding, or Rossmann fold, which contains the active site for adenylate synthesis and for transfer of the aminoacyl moiety from the adenylate to the 3′-end of the bound tRNA (Eriani *et al.*, 1990). This class-defining structural unit is interrupted by one or more insertions which provide residues that interact with the tRNA acceptor stem in order to dock it into the active site (Rould *et al.*, 1989; Nureki *et al.*, 1995; Schimmel and Ribas de Pouplana, 1995). The second domain of the class I synthetase is typically idiosyncratic to the enzyme, and provides for interactions with the second domain of the tRNA, including the anticodon.

Many eukaryote cytoplasmic and mitochondrial class I enzymes present a similar picture, but have one major distinction from their bacterial counterparts. *Saccharomyces cerevisiae* glutaminyl-, methionyl- and isoleucyl-tRNA synthetases, for example, have an additional domain appended to the N- or C-terminal end of the 'body' which itself is closely related to the respective prokaryote enzyme (Mirande, 1991). The four class I enzymes whose structures are solved entirely or in part include *Bacillus stearothermophilus* tyrosyl- (Brick *et al.*, 1988), glutaminyl- (Rould *et al.*, 1989, 1991), *Escherichia coli* methionyl- (Brunie *et al.*, 1990) and tryptophanyl-tRNA synthetases (Doublie *et al.*, 1995). Because these are prokaryote enzymes that lack the appended domain of eukaryote synthetases, no model for the structures of the appended domains is available.

The appended domain of *Neurospora* mitochondrial tyrosyl-tRNA synthetase is required for the novel RNA splicing activity of this synthetase (Cherniack *et al.*, 1990; Mohr *et al.*, 1994). However, more generally the role of the appended domain for enzyme activity is not understood. For example, large deletions in the appended domain of yeast cytoplasmic methionyl-tRNA synthetase yield active protein (Walter *et al.*, 1989). Similarly, large deletions in the appended domain of *S.cerevisiae* cytoplasmic glutaminyl-tRNA synthetase do not compromise the protein's ability to complement a yeast strain containing a deletion of *GLN4*, the gene for the cytoplasmic glutaminyl-tRNA synthetase (Ludmerer and Schimmel, 1987a). In addition, when the domain is excised by mild proteolysis to yield a body essentially corresponding to the *E.coli* protein (Ludmerer *et al.*, 1993), the k_{cat} for aminoacylation and the K_m for tRNA are essentially the same for the truncated protein as for the native enzyme. Thus, the role of the appended domain has remained obscure.

While the body of the yeast enzyme has 40% sequence identity to the *E.coli* synthetase, differences occur at positions critical for the docking of the acceptor stem of $tRNA^{Gln}$ to the *E.coli* glutaminyl-tRNA synthetase (Rould *et al.*, 1989; Jahn *et al.*, 1991). These differences in amino acid sequences correlate with differences in the nucleotide sequences of the respective tRNA acceptor stems, suggesting a species-specific co-adaptation of protein and acceptor stem sequences so that glutamine is faithfully attached to the tRNA bearing its cognate anticodon triplet. For this reason, we anticipated that, in spite of high sequence identity between the two proteins, the selective tRNA sequence differences might prevent the *E.coli* enzyme from charging yeast tRNA. This expectation was confirmed (see below).

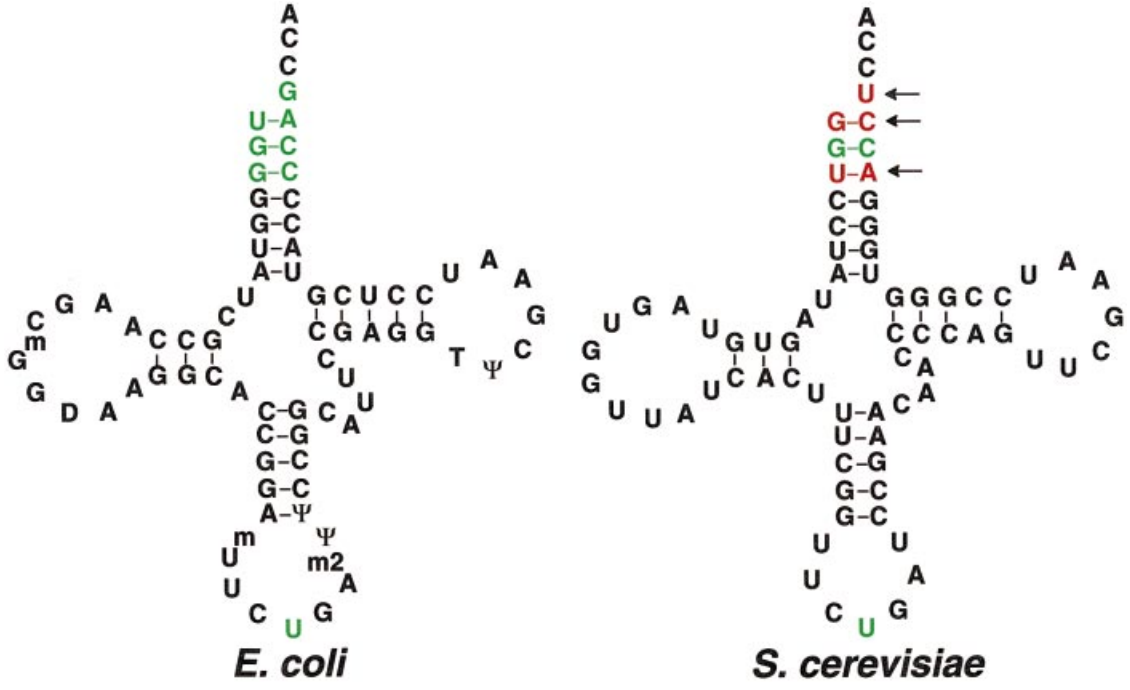

Fig. 1. *Escherichia coli* and yeast glutamine RNA. *Escherichia coli* tRNA^{Gln} (CUG) is shown on the left. Nucleotides important for aminoacylation are highlighted in green (Rould *et al.*, 1989; Jahn *et al.*, 1991). *Saccharomyces cerevisiae* tRNA^{Gln} (CUG) deduced from the gene sequence (Weiss and Friedberg, 1986) and synthesized as a transcript is indicated on the right. Nucleotides that differ from the important *E.coli* tRNA nucleotides are indicated in red and are highlighted with arrows.

This observation motivated us to determine what changes could be made in the *E.coli* protein to enable it to charge yeast tRNA^{Gln}. Given the high sequence similarity between the yeast and *E.coli* enzymes, we viewed the two proteins as homologous and imagined that grafting limited sequences of the yeast into the *E.coli* protein could ultimately give a hybrid *E.coli* enzyme that charged yeast tRNA. Pursuant to this objective, we considered the possibility that a role for the dispensable N-terminal appended domain of the yeast protein might be uncovered by fusing it to the *E.coli* synthetase, without any changes being made to the 'body' of the *E.coli* enzyme. As described below, that fusion transformed the *E.coli* enzyme into a yeast tRNA synthetase that charged yeast tRNA^{Gln}, thus suggesting a previously unanticipated property for the appended N-terminal domain.

Results

Escherichia coli glutaminyl-tRNA synthetase is inactive on yeast tRNA in vitro

The structure of the co-crystal of *E.coli* glutaminyl-tRNA synthetase with tRNA^{Gln} showed that specific tRNA acceptor stem and anticodon nucleotides make contact with the bound protein. Nucleotide substitutions at any of these positions are expected to be deleterious for aminoacylation and their functional significance has been well demonstrated by mutational analyses (Jahn *et al.*, 1991). Because the genetic code is universal, the anticodon triplets for a given tRNA are the same throughout evolution, except for occasional species-specific base modifications that are idiosyncratic to the tRNA and the organism. In comparing tRNA^{Gln} (CUG) from yeast with its counterpart from *E.coli*, the most striking differences at positions important for charging by the *E.coli* enzyme occur in the acceptor stem at the N73 'discriminator base' (Crothers *et al.*, 1972) and the first (1:72) and third (3:70) base pairs (Figure 1). These differences include G73→U, U1–A72→G–C and G3–C70→U–A substitutions in yeast tRNA^{Gln} (CUG). Given these differences, we imagined that the *E.coli* enzyme would not charge yeast tRNA^{Gln} and vice versa.

To test cross-species aminoacylation activity of *E.coli* GlnRS, we used enzyme purified from *E.coli* that was active on *E.coli* tRNA. We challenged this enzyme with yeast tRNA and observed no activity (Figure 2). Conversely, when yeast extracts that were active on yeast tRNA were challenged with *E.coli* tRNA, no aminoacylation of the *E.coli* substrate was observed (data not shown). This result afforded the opportunity to express the *E.coli* enzyme in yeast and see whether extracts of these cells now acquired the ability to charge *E.coli* tRNA. [Given the possibility that *E.coli* GlnRS would be degraded and therefore inactive when produced in yeast, we added a 12CA5 epitope tag (Wilson *et al.*, 1984) to the C-terminus of the protein to facilitate detection by Western blot methods. This tag did not disrupt the activity of the *E.coli* protein and the expected apparent mol. wt (65 kDa) was observed (data not shown).] When the *E.coli* enzyme was expressed in yeast, extracts of these cells now charged *E.coli* tRNA (Figure 2).

These experiments showed that each enzyme has aminoacylation activity that is strictly species specific. The lack of charging of yeast tRNA by *E.coli* GlnRS suggested that a yeast strain defective in yeast GlnRS would not be rescued by the *E.coli* enzyme. In addition, the apparent

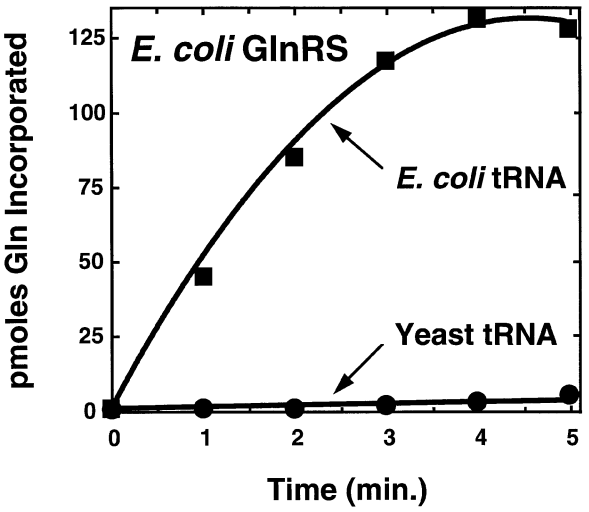

Fig. 2. Aminoacylation at pH 7.5, ambient temperature of 0.16 mM *E.coli* tRNA (squares) by extracts of yeast cells [10 µl (~10 µg)] expressing *E.coli* GlnRS which contains a 12CA5 epitope at the C-terminus. (Without expression of *E.coli* GlnRS, there is no aminoacylation of *E.coli* tRNA by the yeast extracts.) Aminoacylation of 0.1 mM yeast tRNA (circles) with 20 nM native *E.coli* GlnRS purified from *E.coli* (Hoben *et al.*, 1982).

stability of the *E.coli* enzyme when expressed in yeast made it feasible to investigate which alterations in *E.coli* GlnRS were sufficient to enable it to rescue a GlnRS-deficient yeast strain.

Escherichia coli GlnRS fails to rescue a GlnRS-deficient yeast strain

An 835-bp *Eco*RI fragment of the gene *GLN4* for cytoplasmic glutaminyl-tRNA synthetase was deleted and replaced by *TRP1*, using standard genetic methods with a *ura3*$^-$ strain (Guthrie and Fink, 1991). The deletion removed codons 384–662 from the 809 amino acid yeast enzyme. This deleted a large portion of the sequence coding for the active-site-containing nucleotide binding fold that extends from codons 253 to 500. The resulting strain was designated EFW6.

The EFW6 *gln4*Δ*::TRP1* strain is maintained by plasmid pEFW111 which contains *GLN4* and the selectable marker *URA3*. When a second plasmid containing a functional GlnRS and a different selectable marker (*LEU2*) is introduced, the pEFW111 maintenance plasmid is lost by growth on 5-fluoroorotic acid (5-FOA) (Sikorski and Hieter, 1989). The *E.coli* glutaminyl-tRNA synthetase gene (*glnS*) [with the engineered 12CA5 epitope (see above)] was cloned into the high copy plasmid pDB20L (Berger *et al.*, 1992), in which expression is driven by the strong constitutive alcohol dehydrogenase (ADH) promoter. While expression of the yeast glutaminyl-tRNA synthetase cloned into plasmid pRS315 (Sikorski and Hieter, 1989) resulted in complementation of EFW6, expression of the *E.coli* protein did not rescue the lethal phenotype caused by the knock-out mutation (Figure 3). We established (by Western blot analysis using the 12CA5 epitope) that the *E.coli* enzyme was overproduced in yeast and confirmed that the *E.coli* enzyme expressed in and isolated from yeast charged its cognate *E.coli* tRNA substrate (cf. Figure 2). The failure of the *E.coli* enzyme to complement the yeast *GLN4* disruption strain was consistent with the inability of the enzyme to charge yeast tRNA *in vitro*.

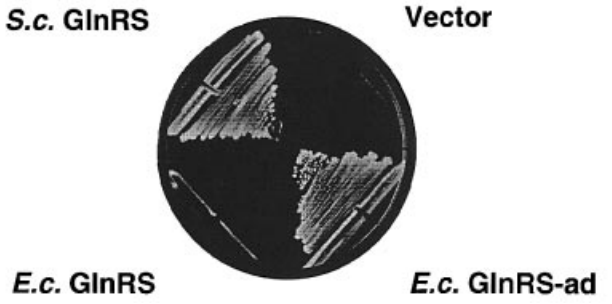

Fig. 3. EFW6 rescued by *E.coli* fusion enzyme. Growth at 30°C of the yeast *gln4*Δ*::TRP1* strain EFW6 on 5-FOA plates. Cells expressing the *E.coli* enzyme containing a 12CA5 epitope or containing just the expression vector do not grow. In contrast, expression of yeast GlnRS or of *E.coli* GlnRS-ad containing a 12CA5 epitope rescued cell growth.

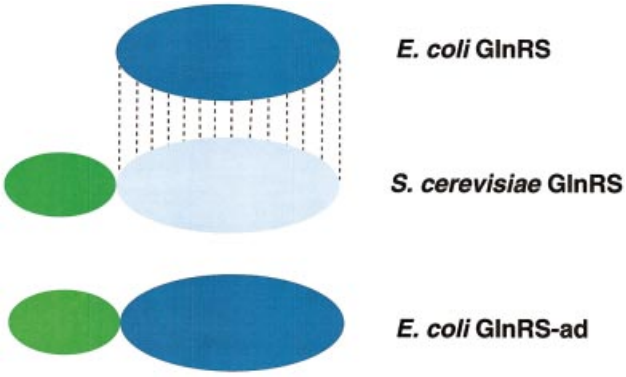

Fig. 4. Schematic diagram denoting the yeast and *E.coli* glutaminyl-tRNA synthetases. *Escherichia coli* GlnRS is shown in dark blue which aligns with the core of the *S.cerevisiae* GlnRS shown in light blue. The aligned region is indicated by dashed lines. The appended domain is shown in green. The fused enzyme, *E.coli* GlnRS-ad, contains the yeast appended domain fused to the full-length *E.coli* GlnRS.

Escherichia coli GlnRS fused to appended domain of yeast enzyme complements the GlnRS-deficient yeast strain

Escherichia coli GlnRS is a 551 amino acid monomeric enzyme, while *S.cerevisiae* cytoplasmic GlnRS is an 809 amino acid monomer that, starting at amino acid 230, aligns with the N-terminus of the *E.coli* enzyme (Ludmerer and Schimmel, 1987b; Lamour *et al.*, 1994) (Figure 4). From that point, the sequences of the two enzymes have a 40% sequence identity with a few small gaps to maintain the alignment. In portions of the active site, the alignment is so strong that as many as 15 consecutive amino acids are identical (Ludmerer and Schimmel, 1987b). Almost all of the extra length of the yeast protein is due to the 229 amino acid appended domain at the N-terminus (Lamour *et al.*, 1994).

We fused the 229 amino acid appended domain (ad) of the yeast protein to the N-terminus of *E.coli* glutaminyl-tRNA synthetase to give the fusion protein *E.coli* GlnRS-ad (Figure 4). The gene for the fusion protein was cloned into the low copy plasmid pRS315 (Sikorski and Hieter, 1989) where expression was driven by the *GLN4* promoter.

[The fusion protein contained the same 12CA5 epitope at the C-terminus as that joined to the unfused, native *E.coli* enzyme (see above).] Expression of *E.coli* GlnRS-ad rescued the lethal phenotype of EFW6 on 5-FOA (Figure 3). The same complementation phenotype was obtained when the fusion protein was expressed behind the ADH promoter in the high copy plasmid pDB20L (data not shown).

In these experiments, retention of the deletion/disruption of EFW6 was shown by the $Leu^+Trp^+Ura^-$ phenotype of the cells complemented by *E.coli* GlnRS-ad. This showed that *GLN4* was disrupted with *TRP1* and that the pEFW111 maintenance plasmid was lost. Furthermore, plasmid isolated from the complementation plate was verified (by restriction mapping) to contain the gene encoding the fusion protein. Finally, immunoblot analysis using the 12CA5 epitope confirmed expression of the *E.coli* GlnRS-ad protein with an apparent mol. wt of 91 kDa (data not shown). Thus, even though *E.coli* GlnRS was inactive on yeast tRNA, *E.coli* GlnRS-ad served as the sole source of glutaminyl-tRNA synthetase activity in *S.cerevisiae*.

Proteins encoding large deletions in the N-terminal extension of yeast GlnRS were reported previously to complement a yeast strain harboring a different knockout allele of *GLN4* than the one used here (Ludmerer and Schimmel, 1987a). In this work, we obtained a similar result using a plasmid encoding a deletion of codons 75–199 of the appended domain of *GLN4*, with the EFW6 *gln4Δ::TRP1* strain used in the present study. Expression of the internally deleted (75–199) *GLN4* in EFW6 resulted in growth complementation. However, when the same internal deletion of the appended domain was placed in *E.coli* GlnRS-ad, the resulting protein was unstable in yeast and no complementation was observed (data not shown). Thus, the appended domain is sensitive to the origin of the activity-containing 'body' to which it is joined.

To determine whether an arbitrary domain added to the N-terminus of the *E.coli* protein could rescue the lethal phenotype of EFW6, we fused glutathione-*S*-transferase (GST) (Smith and Johnson, 1988) to the *E.coli* enzyme. The GST extension is similar in size to the appended domain of yeast glutaminyl-tRNA synthetase. (The GST fusion added 239 residues to the *E.coli* enzyme.) Although this GST fusion enzyme was expressed as a stable protein (apparent mol. wt, 91 kDa) and accumulated in yeast, it did not complement EFW6 (data not shown).

Escherichia coli GlnRS-ad is active on yeast tRNA in vitro

The complementation results imply that *E.coli* GlnRS-ad is active on *S.cerevisiae* tRNA. To investigate this activity *in vitro*, two approaches were used. First, we tested the ability of *E.coli* GlnRS-ad isolated from the rescued yeast deletion strain EFW6 to charge yeast tRNA. Yeast lysates expressing *E.coli* GlnRS-ad aminoacylated yeast tRNA and, in addition, charged a yeast $tRNA^{Gln}$ transcript (data not shown). These encouraging results with crude yeast lysates motivated us to check heterologous aminoacylation further by working with purified *E.coli* GlnRS-ad.

For this purpose, we joined a His6-tag to the C-terminus of the 12CA5 epitope-tagged *E.coli* GlnRS-ad. We joined the same His6-tag to the 12CA5 epitope-tagged *E.coli* GlnRS. Both proteins were expressed in *E.coli*, isolated on a Ni–NTA affinity column, and purified to homogeneity. Their N-terminal sequences were determined in the MIT Biopolymers Laboratory and each matched that predicted by the respective DNA sequence. Working with the purified proteins, we found that *E.coli* GlnRS-ad was active on yeast tRNA and that *E.coli* GlnRS was not (Figure 5). The same results were obtained when a yeast tRNAGln transcript was tested (data not shown).

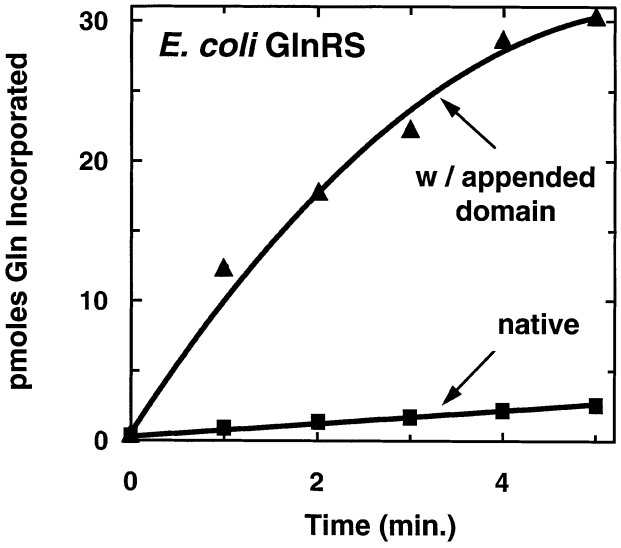

Fig. 5. Aminoacylation at pH 7.5, ambient temperature of 0.1 mM yeast tRNA by 20 nM His_6-tagged *E.coli* GlnRS-ad (triangles) and 20 nM His_6-tagged *E.coli* GlnRS (squares) purified from *E.coli* cells. Analogous results were obtained when a yeast $tRNA^{Gln}$ transcript (3 μM) was aminoacylated with these purified enzymes.

The yeast appended domain enables E.coli GlnRS to bind to yeast glutamine tRNA

The experiments described above do not address the question of whether the inability of *E.coli* GlnRS to charge yeast tRNA is due to a failure to bind the yeast substrate or, alternatively, whether a synthetase–tRNA complex forms without being able to create the transition state for aminoacylation. To investigate whether the appended domain specifically affected tRNA binding, the nitrocellulose filter assay was used to measure association between yeast $tRNA^{Gln}$ and *E.coli* GlnRS, with and without the appended domain of yeast glutaminyl-tRNA synthetase. These experiments showed that, while the purified *E.coli* enzyme has little or no detectable binding to yeast $tRNA^{Gln}$, addition of the appended domain conferred binding activity (Figure 6). From these experiments, we estimate the dissociation constant at pH 7.5 for the *E.coli* GlnRS-ad–yeast tRNAGln (CUG) complex to be 0.5–1.0 μM. This value is similar to the K_m (at pH 7.5, 37°C) of 0.5 μM for *E.coli* GlnRS with *E.coli* $tRNA^{Gln}$ (Jahn *et al.*, 1991) and of 1.7 μM (pH 7.5, 30°C) for yeast GlnRS with yeast $tRNA^{Gln}$ (Ludmerer *et al.*, 1993).

The tRNA binding activity of *E.coli* GlnRS-ad was not highly specific, however, because we also detected binding of *E.coli* GlnRS-ad to *E.coli* $tRNA^{Glu}$ and to $tRNA^{Ile}$ (data not shown). Thus, the appended domain may have general RNA binding properties that act in cooperation with the highly specific tRNA docking site in the body of the

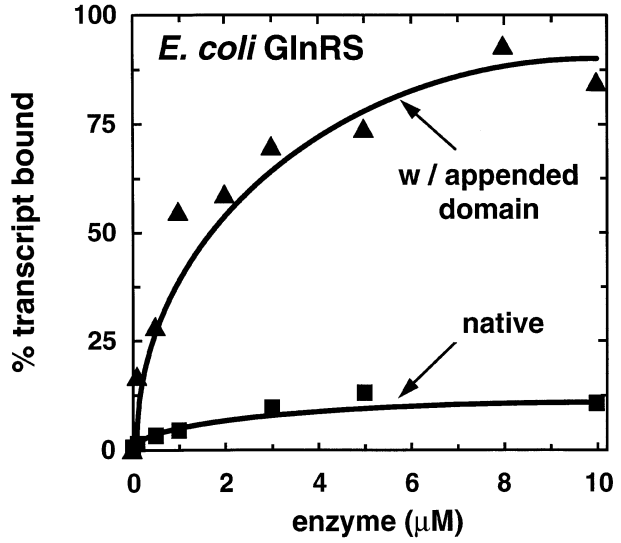

Fig. 6. Filter assay (pH 7.5, ambient temperature) for binding of a yeast $tRNA^{Gln}$ transcript (~5 nM) to 0–10 μM of the His_6-tagged *E.coli* GlnRS-ad (triangles) and His_6-tagged *E.coli* GlnRS (squares).

enzyme. However, *E.coli* GlnRS-ad did not charge *E.coli* $tRNA^{Glu}$ or $tRNA^{Ile}$ (data not shown). [To test the possibility that the appended domain has general RNA binding properties, we attempted to express and isolate it as a free protein (with the 12CA5 tag). This attempt failed, apparently because the expressed domain was unstable.] This result is consistent with *E.coli* GlnRS-ad rescuing the yeast knock-out strain EFW6. If *E.coli* GlnRS-ad catalyzed a significant amount of misacylation, then work on other systems suggests that toxicity would result (Inokuchi *et al.*, 1984; Bedouelle *et al.*, 1990; Vidal-Cros and Bedouelle, 1992).

Escherichia coli GlnRS-ad preferentially charges E.coli versus yeast tRNA

Because *E.coli* glutaminyl-tRNA synthetase is not active on yeast tRNA (Figure 2), we wondered whether fusion of the appended domain resulted in an enzyme that still had at least some discrimination between *E.coli* and yeast tRNA. To address this question, we used an equal amount of *E.coli* GlnRS-ad with tRNA samples that were estimated to have the same concentrations of glutamine-specific yeast or *E.coli* glutamine acceptors. In repeated experiments, *E.coli* GlnRS-ad was observed to have substantially higher activity on *E.coli* tRNA. We estimated that k_{cat}/K_m for *E.coli* tRNA was ~30-fold higher than that for yeast tRNA. This difference corresponds to ~2 kcal/mol of transition state free energy of stabilization for *E.coli* GlnRS-ad with the *E.coli* versus the yeast tRNA substrate.

Discussion

The appended domain of yeast glutaminyl-tRNA synthetase rescues a defective synthetase–tRNA interaction while maintaining specificity for aminoacylation. Given the high sequence identity of the 'bodies' of the yeast and *E.coli* proteins, we imagine that they are folded into a closely similar three-dimensional structure. The yeast and *E.coli* glutamine tRNAs are conventional molecules that fold into the same three dimensional structure that is represented by that of yeast $tRNA^{Phe}$ (Kim *et al.*, 1974; Robertus *et al.*, 1974). Thus, the productive complexes between yeast GlnRS or *E.coli* GlnRS-ad with yeast $tRNA^{Gln}$ are likely to dock the tRNA structure on the synthetase in the same way as that seen for the crystal structure of *E.coli* GlnRS with *E.coli* $tRNA^{Gln}$.

The affinity of tRNAs for their cognate synthetases is generally characterized by dissociation constants of the order of 0.1–1 μM under physiological conditions (Schimmel and Soll, 1979; Giege *et al.*, 1993). The relatively weak nature of these complexes assures that the enzymes turn over rapidly during aminoacylation and protein synthesis. However, the modest synthetase–tRNA dissociation constants are insufficient by themselves to account for the specificity of synthetase aminoacylation of tRNAs. In addition to the binding interactions, the transition state of catalysis (k_{cat} discrimination) has an important role in determining specificity. Thus, a non-cognate, mutant tRNA may bind competitively to the same site on a synthetase as the wild-type tRNA substrate, but not be charged (Schimmel and Soll, 1979; Park *et al.*, 1989).

From the perspective of the *E.coli* protein, yeast $tRNA^{Gln}$ can be viewed as a mutant tRNA with multiple substitutions at sites critical for recognition (Figure 1). In our experiments, the appended domain increased the apparent affinity of *E.coli* GlnRS for yeast tRNA (Figure 6). In addition, the bound yeast tRNA substrate is charged by the *E.coli* enzyme that is joined to the yeast appended domain. This result is particularly striking because it shows that binding of yeast $tRNA^{Gln}$ to *E.coli* GlnRS-ad forms an active transition state complex and not the abortive complex that is often seen with mutant tRNAs. This observation is strong evidence that the *E.coli* enzyme forms a complex essentially identical to that seen with its normal *E.coli* $tRNA^{Gln}$ substrate. That is, the appended domain overcomes the deleterious nucleotide replacements found in yeast $tRNA^{Gln}$.

The natural role of the appended domain of yeast glutaminyl-tRNA synthetase remains unclear. While the existence of a multi-synthetase complex in yeast is controversial (Mirande, 1991; Harris and Kolanko, 1995), for some tRNA synthetases in higher eukaryotes an extra domain is believed important for formation of multi-synthetase complexes (Mirande, 1991; Kerjan *et al.*, 1994; Barbarese *et al.*, 1995; Rho *et al.*, 1996). Database searches revealed that many yeast synthetases have an extra appended domain that, like yeast GlnRS, is lysine-rich (Mirande, 1991; E.F.Whelihan and P.Schimmel, unpublished data). Similarly, a number of mammalian tRNA synthetases have an extra appended domain of unknown function (K.Shiba and P.Schimmel, unpublished data). These include the threonyl-, cysteinyl-, asparaginyl-, seryl-, histidyl-, tryptophanyl- and glycyl-tRNA synthetases, all of which are believed not to be part of the multi-synthetase complex.

Our results suggest that one role for these domains could be to enhance the synthetase–tRNA interaction, but only in circumstances where interaction along the normal enzyme–tRNA interface is weakened, either by mutation or by the local cellular environment. Thus, in its natural role as a domain fused to yeast GlnRS, it may switch between a 'bound' and an 'unbound' conformation, and

only be in the 'bound' form when contacts of the body of the enzyme with the tRNA have been weakened. [This kind of switch would explain why the K_m of yeast GlnRS for $tRNA^{Gln}$, measured under native conditions, is essentially unaffected by the appended domain (Ludmerer *et al.*, 1993).] In this way, the appended domain could act as a buffer against changes that would otherwise perturb an essential protein–RNA complex. In addition, these domains might also be adapted for specialized RNA-related function in specific instances, such as that seen with *Neurospora* mitochondrial tyrosyl-tRNA synthetase (Cherniack *et al.*, 1990; Mohr *et al.*, 1994) and that recently proposed for yeast cytoplasmic methionyl-tRNA synthetase (Simos *et al.*, 1996).

Materials and methods

Aminoacylation assays and substrates

Aminoacylation activity was assayed at ambient temperature (~23°C) in the following buffer: 30 mM HEPES (pH 7.5), 25 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 4 mM ATP and 300 μM glutamine (5 μM [^{3}H]glutamine; Amersham, Arlington Heights, IL). Yeast lysate [10 μl (~10 μg)] expressing *E.coli* GlnRS, epitope-tagged at the C-terminus with 12CA5 (described below), or 20 nM native *E.coli* GlnRS (Hoben *et al.*, 1982) was assayed with 0.1–0.6 mM *E.coli* or brewer's yeast tRNA (Boehringer Mannheim, Indianapolis, IN). Yeast lysate [10 μl (~10 μg)] expressing *E.coli* GlnRS-ad, epitope-tagged at the C-terminus with 12CA5 (described below), was assayed with 0.1 mM brewer's yeast tRNA.

Yeast $tRNA^{Gln}$ (CUG) was cloned from genomic yeast DNA by PCR. The T7 promoter was introduced at the 5′-end and a *Bst*NI site at the 3′-end of the gene. *In vitro* run-off transcription of the *Bst*NI linearized DNA was performed with the Stratagene RNA polymerase kit (Stratagene, La Jolla, CA). The resulting transcript was purified by denaturing polyacrylamide gel electrophoresis (12%, 29:1 bis:acrylamide), excised from the gel, and passively eluted from the acrylamide at 37°C with standard elution buffer (0.5 M NH_4OAc pH 7.5, 1 mM EDTA). The transcript was refolded (3 μM) prior to use in aminoacylation assays.

Deletion and disruption of GLN4

Saccharomyces cerevisiae strain MM1401 (*MAT***a**/α, *ade2-101/+, can1/+, his3Δ200/his3Δ200, leu2Δ1/leu2Δ1, lys2-801/lys2-801, trp1Δ101/trp1Δ101, ura3-52/ura3-52*), obtained from John Woolford (Carnegie Mellon University, Pittsburg, PA), was the diploid strain used to disrupt the *GLN4* gene. Standard genetic techniques were employed (Guthrie and Fink, 1991). Yeast cells were grown in YPD (1% Difco-yeast extract, 2% Difco-peptone, 2% glucose) or defined synthetic medium supplemented with 2% glucose as a carbon source. A *gln4* partial deletion was created by replacing the 835-bp *Eco*RI restriction fragment of *GLN4* coding sequence with the *TRP1* gene. This deletion removed residues 384–662 from the 809 amino acid full-length protein and thereby took out a large portion of the active site. Trp^+ colonies were selected after transformation of strain MM1401 with an *Eag*I–*Xho*I restriction fragment containing the *gln4Δ::TRP1* disruption.

The expected transplacement was confirmed by genomic Southern blotting (Sambrook *et al.*, 1989) (data not shown). Diploids were sporulated and tetrads dissected. Only two spores were viable in each tetrad and each was Trp^-. Subclones of yeast *GLN4*, *E.coli glnS* and the gene encoding *E.coli* GlnRS-ad were inserted into high copy and low copy pRS vectors (Sikorski and Hieter, 1989) as well as pDB20L (which contains the ADH promoter) (Berger *et al.*, 1992). A plasmid bearing *GLN4* and the selectable marker *URA3* (pEFW111) was constructed by subcloning a *Stu*I–*Eco*RV restriction fragment of *GLN4* into the *Sma*I site of pRS316 and was used to transform the *gln4Δ::TRP1/GLN4* diploid and the transformants were sporulated. Trp^+ spores were recovered, all of which were Ura^+, thus indicating that *GLN4* is essential as was originally reported (Ludmerer and Schimmel, 1987a). The resulting *gln4Δ::TRP1* haploid strain which is maintained by pEFW111 is designated as EFW6.

Addition of epitope tag to E.coli GlnRS

*Not*I sites were introduced at each terminus of *glnS* to subclone the *E.coli* gene into pDB20L (Berger *et al.*, 1992) and a 12CA5 epitope (from the influenza hemagglutinin protein) was introduced via loop-out mutagenesis (Sambrook *et al.*, 1989) so that Western blot analysis utilizing anti-12CA5 antibodies would detect protein expression (Wilson *et al.*, 1984). A synthetic 66 nucleotide primer was used to introduce a 12CA5 epitope three codons prior to the stop codon. The synthetic nucleotide contained the epitope coding sequence flanked by 18 nucleotides that are complementary to DNA on the 5′-end of the insertion site and 21 nucleotides complementary to the 3′-side. Incorporation of the epitope tag was verified by introduction of a *Hin*dIII restriction site and by DNA dideoxy sequencing.

Construction of E.coli GlnRS-ad and of a GST fusion of E.coli GlnRS

*Nde*I sites were introduced (Kunkel, 1985) in the gene for the yeast enzyme and at the start of *E.coli glnS* to construct the gene for *E.coli* GlnRS-ad, which consists of the yeast appended domain (residues 1–229) fused to full-length *E.coli* GlnRS. This construct also contained the 12CA5 epitope tag described above.

GST was cloned by PCR and the *Sph*I and *Nde*I sites were introduced at the 5′- and 3′-termini of the gene. Fusion of this fragment to the 5′-*Nde*I modified *E.coli* gene described above resulted in a GST–*E.coli* GlnRS fusion.

Construction and purification of His_6-tagged enzymes

*Sph*I and *Bam*HI sites were introduced at the 5′- and 3′-termini, respectively, of the gene encoding *E.coli* GlnRS-ad using site-directed mutagenesis (Kunkel, 1985). The same sites were introduced at the termini of *glnS*. Each construct contains the 12CA5 epitope upstream of the C-terminal His_6-tag. The resulting DNAs were independently subcloned into the Qiagen pQE70 vector and standard protocols were used for expression and for purification on a Ni–NTA affinity column (Qiagen, Chatsworth, CA). To remove degradation products, the His_6-tagged *E.coli* GlnRS-ad was further purified on a Mono-S column (Pharmacia, Piscataway, NJ) following the protocol for yeast GlnRS (Ludmerer *et al.*, 1993). Aminoacylation conditions were as described above. His_6-tagged *E.coli* GlnRS-ad (20 nM) and *E.coli* GlnRS (20 nM) were assayed with brewer's yeast tRNA (0.1 mM) and yeast $tRNA^{Gln}$ (CUG) transcript (3 μM).

Nitrocellulose filter binding assays

Nitrocellulose filter binding assay was used to measure the binding affinity of His_6-tagged *E.coli* GlnRS-ad (0–10 μM) and His_6-tagged *E.coli* GlnRS (0–10 μM) to yeast $tRNA^{Gln}$ (CUG) transcript (~5 nM). Standard procedures for protein–DNA binding were followed (Ausubel *et al.*, 1989), except that diethyl-pyrocarbonate-treated water (Sambrook *et al.*, 1989) was used throughout and the binding and elution buffers were the same [20 mM Tris–HCl (pH 7.5), 10% glycerol, 50 mM KCl, 0.1 mM DTT]. Yeast transcript was internally labeled with [α-^{32}P]rUTP (400 Ci/mmol, Amersham) using the Stratagene RNA Transcription Kit. RNasin ribonuclease inhibitor (1 μl, 40 U/μl Promega, Madison, WI) and cold UTP (8 μM) was added to enhance polymerase activity. The radiolabeled product was purified as described above for the unlabeled transcript.

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