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.

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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 (Doublet 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 kcat for aminoaacylation and the Kcat 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\(^{\text{Gln}}\) (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\(^{\text{Gln}}\) (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\(^{\text{Gln}}\). 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\(^{\text{Gln}}\), 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\(^{\text{Gln}}\) 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\(^{\text{Gln}}\) (CUG) from yeast with its counter-part 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\(^{\text{Gln}}\) (CUG). Given these differences, we imagined that the *E. coli* enzyme would not charge yeast tRNA\(^{\text{Gln}}\) and 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\(^{\text{Gln}}\), thus suggesting a previously unanticipated property for the appended N-terminal domain.

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
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 EcoRI 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 *gln4Δ: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* glutaminyltRNA 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 glutaminyltRNA 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.

**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* glutaminyltRNA 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 E FW6 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<sub>Glu</sub> (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-ad, the resulting protein was unstable in internal deletion of the appended domain was placed in active on yeast tRNA 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*.

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**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<sup>Glu</sup> 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<sup>Glu</sup>, 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 tRNA<sup>Glu</sup> complex to be 0.5–1.0 μM. This value is similar to the *K<sub>m</sub>* (at pH 7.5, 37°C) of 0.5 μM for *E. coli* GlnRS with *E. coli* tRNA<sub>Glu</sub> (Jahn et al., 1991) and of 1.7 μM (pH 7.5, 30°C) for yeast GlnRS with yeast tRNA<sup>Glu</sup> (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<sup>Glu</sup> and to tRNA<sup>Asp</sup> (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 protein.
enzyme. However, *E. coli* GlnRS-ad did not charge *E. coli* tRNA<sup>Glu</sup> or tRNA<sup>Leu</sup> (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<sup>Phe</sup> (Kim et al., 1974; Robertus et al., 1974). Thus, the productive complexes between yeast GlnRS or *E. coli* GlnRS-ad with yeast tRNA<sup>Glu</sup> 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<sup>Glu</sup>.

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<sup>Glu</sup> 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<sup>Glu</sup> 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<sup>Glu</sup> substrate. That is, the appended domain overcomes the deleterious nucleotide replacements found in yeast tRNA<sup>Glu</sup>.

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; Barbares 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 glycy1-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, 13 mM MgCl$_2$, 5 mM DTT, 4 mM ATP and 300 μM glutamine (5 μM [3H]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 RNA (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$^{Lys}$ (CUG) was cloned from genomic yeast DNA by PCR. The T7 promoter was introduced at the 5’-end and a BoN1 site at the 3’-end of the gene. In vitro run-off transcription of the BoN1 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% biacrylamide), excised from the gel, and passively eluted from the acrylamide at 37°C with standard elution buffer (0.5 M NH$_4$OAc 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 (MATaα, ade-2-101/+; can1/+, his3Δ200/his3Δ200, leu2Δ1/leu2Δ1, lys2-801/lys2-801, trplΔ101/trplΔ101, ura3-52/ura3-52), obtained from John Wooldford (Carnegie Mellon University, Pittsburgh, PA), was the diploid strain used to disrupt the GLN4 gene. Standard genetic techniques were employed (Guthrie (400 Ci/mmol, Amersham) using the Stratagene RNA Transcription Kit. Yeast transcript was internally labeled with [α-32P]rUTP (4000 Ci/mmol, Amersham) using the Stratagene RNA Transcription Kit. RNasin ribonuclease inhibitor (1 μl, 40 U) (Promega, Madison, WI) and cold UTP (8 μM) was added to enhance polymerase activity. The radiolabeled transcript was purified as described above for the unlabeled transcript.

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References


Addition of epitope tag to E.coli GlnRS

No1 sites were introduced at each terminal 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 this way, 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 HindIII restriction site and by DNA dyeoxy sequencing.

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

No1 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 Spfl and Ndel sites were introduced at the 5’- and 3’-termini of the gene. Fusion of this fragment to the 5’-Ndel modified E.coli gene described above resulted in a GST–E.coli GlnRS fusion.

Construction and purification of His$_6$-tagged enzymes

Spfl and BamHI 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 terminal of glnS. Each construct contains the 12CA5 epitope upstream of the C-terminal His$_6$-tag. The resulting DNAs were independently sub-cloned 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 μM) and E.coli GlnRS (20 μM) 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 μM). Standard procedures for protein–DNA binding were followed (Ausubel et al., 1989), except that diethylpyrocarbonate-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 [3H]glutamine; Amersham, Arlington Heights, IL). Yeast lysate [10 μl (~10 μg)] expressing E.coli GlnRS (0–10 μM) were assayed with brewer’s yeast tRNA (0.1 mM) and yeast tRNA$^{Gln}$ (CUG) transcript (3 μM).