

Characterization of an 'orthogonal' suppressor tRNA derived from *E. coli* tRNA₂^{Gln}

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Background: In an effort to expand further our ability to manipulate protein structure, we have completed the first step towards a general method that allows the site-specific incorporation of unnatural amino acids into proteins *in vivo*. Our approach involves the construction of an 'orthogonal' suppressor tRNA that is uniquely acylated *in vivo*, by an engineered aminoacyl-tRNA synthetase, with the desired unnatural amino acid. The *Escherichia coli* tRNA₂^{Gln}-glutaminyl-tRNA synthetase (GlnRS) pair provides a biochemically and structurally well-characterized starting point for developing this methodology. To generate the orthogonal tRNA, mutations were introduced into the acceptor stem, D-loop/stem, and anticodon loop of tRNA₂^{Gln}. We report here the characterization of the properties of the resulting tRNAs and their suitability to serve as an orthogonal suppressor. Our efforts to generate an engineered synthetase are described elsewhere.

Results: Mutant tRNAs were generated by runoff transcription and assayed for their ability to be aminoacylated by purified *E. coli* GlnRS and to suppress an amber codon in an *in vitro* transcription/translation reaction. One tRNA bearing eight mutations satisfies the minimal requirements for the delivery of an unnatural amino acid: it is not acylated by any endogenous *E. coli* aminoacyl-tRNA synthetase, including GlnRS, yet functions efficiently during protein translation. Mutations in the acceptor stem and D-loop/stem, when introduced in combination, had very different effects on the properties of the resulting tRNAs compared with the effects of the individual mutations.

Conclusions: Mutations at sites within tRNA₂^{Gln} separated by 23–31 Å interact strongly with each other, often in a nonadditive fashion, to modulate both aminoacylation activities and translational efficiencies. The observed correlation between the effects of mutations at very distinct regions of the GlnRS-tRNA and possibly the ribosomal/tRNA complexes may contribute in part to the fidelity of protein biosynthesis.

Introduction

An *in vitro* protein mutagenesis method that involves suppression of amber codons with chemically aminoacylated suppressor tRNAs has been used to selectively incorporate a wide variety of unnatural amino acids into proteins [1–3]. We are currently attempting to expand the scope of this approach to allow the site-specific incorporation of unnatural amino acids into proteins *in vivo*, directly from the growth medium, obviating the need for chemically aminoacylated tRNAs [4]. This methodology would allow the generation of large quantities of proteins containing fluorophores, spin labels, photoactivatable groups and novel chemical functionality. In addition, the function of these modified proteins could be studied both *in vitro* and *in vivo*.

Our approach requires the generation of an 'orthogonal' suppressor tRNA that is not a substrate for any endogenous aminoacyl-tRNA synthetase but that also functions

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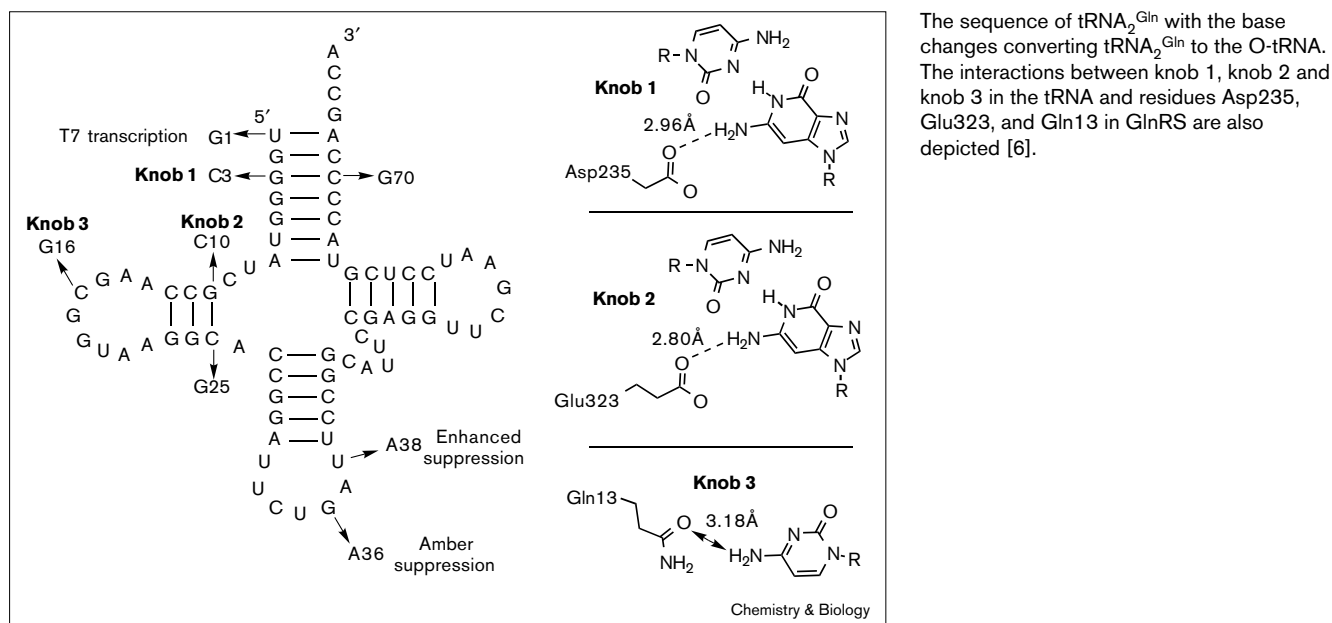
efficiently in translation. An aminoacyl-tRNA synthetase must then be evolved that uniquely acylates the O-tRNA with the desired unnatural amino acid but not with a common amino acid [5]. In the course of our recent efforts to engineer an orthogonal tRNA from *Escherichia coli* tRNA₂^{Gln} [5], we have generated several suppressor tRNAs bearing a variety of combinations of mutations. We report here the characterization of these mutant tRNAs with *E. coli* glutaminyl-tRNA synthetase (GlnRS), other endogenous *E. coli* aminoacyl-tRNA synthetases and the protein biosynthetic machinery.

Results

Design of an orthogonal tRNA

The generation of an orthogonal tRNA-aminoacyl-tRNA synthetase pair capable of selectively incorporating unnatural amino acids into proteins *in vivo* will probably require significant modification of an existing tRNA-synthetase

Figure 1



pair, both at the tRNA–enzyme interface and in the active site. Structural information will provide important guidance to this process. Consequently, we have focused our efforts on the structurally and biochemically well-characterized *E. coli* GlnRS–tRNA₂^{Gln} pair [6,7]. Because *E. coli* is being used initially as the host organism to simplify genetic manipulations, the orthogonal tRNA must not be aminoacylated by any endogenous *E. coli* aminoacyl-tRNA synthetase yet it must function efficiently in the *E. coli* protein translational machinery.

On the basis of an analysis of the three-dimensional X-ray crystal structure of *E. coli* GlnRS complexed with tRNA₂^{Gln} [6], three potentially critical contacts ('knobs') at the protein–tRNA interface were identified [5]. These include (Figure 1) the exocyclic amine of base G3 hydrogen bonding with the carboxylate of Asp235 (knob 1); the exocyclic amine of base G10 hydrogen bonding with the carboxylate of Glu323 (knob 2); and the exocyclic amine of base C16 contacting the carboxamide sidechain of Gln13 (knob 3). We hypothesized that mutations at these sites that preserve base-pairing interactions could eliminate acylation of the resulting tRNAs by GlnRS while maintaining the structural aspects of the tRNAs required for efficient translation. Indeed, previous mutations at these three sites in the tRNA resulted in 2–400-fold decreases in k_{cat}/K_m of GlnRS for the mutant tRNAs [8–10]. In the case of knob 1, we changed the base pair G3–C70 to C3–G70; in the case of knob 2, base pair G10–C25 was changed to C10–G25; and at knob 3, the pyrimidine C16 was changed to a purine (G16). In addition, the tRNA anticodon was changed from CUG to

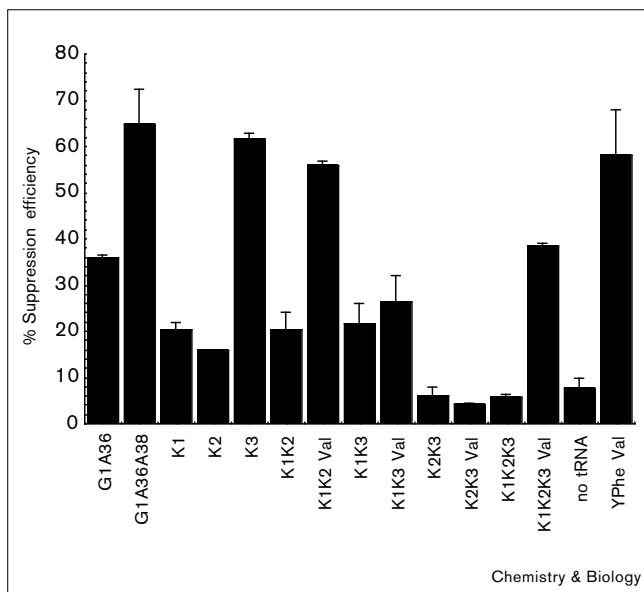
CUA to allow amber suppression, the first base of the tRNA was changed from U1 to G1 to allow efficient T7 RNA polymerase transcription and to provide the possibility of further discrimination by the wild-type GlnRS, and base U38 was mutated to A38 to enhance suppression efficiency (Figure 1) [11].

Suppression efficiencies of mutant tRNAs

The mutant tRNA genes containing all possible combinations of knobs 1, 2 and 3 were constructed by primer extension from overlapping oligonucleotides and placed behind a T7 RNA polymerase promoter. Full-length tRNAs were generated by runoff transcription of *Bst*NI-digested templates. Each tRNA was then assayed for its ability to suppress an amber codon at position 88 of *E. coli* chorismate mutase [12] when added to an *in vitro* transcription and translation reaction containing all soluble cellular proteins, including aminoacyl-tRNA synthetases. A low suppression efficiency, measured by the amount of full-length chorismate mutase produced in the reaction, indicates that the suppressor tRNA is not efficiently acylated by any endogenous aminoacyl-tRNA synthetase, is not efficiently accepted by the ribosomal machinery or both. The results of these experiments are summarized in Figure 2.

The mutant suppressor tRNA₂^{Gln}(G1 A36) afforded a 36% suppression efficiency when added to the *in vitro* transcription and translation reaction described above (Figure 2). This is consistent with the *in vivo* behavior of the *supE* suppressor tRNA₂^{Gln}(A36) which typically yields 10–30% suppressed protein product [13], even though the

Figure 2



A comparison of the suppression efficiencies of tRNA₂^{Gln}-derived suppressor tRNAs in *E. coli* chorismate mutase at site Gln88. Suppression efficiencies (two independent trials per tRNA) are defined as the amount of full-length protein divided by the sum of the full-length and truncated protein produced in each reaction. The tRNAs are identified below each bar: G1A36, tRNA₂^{Gln}(G1 A36); G1A36A38, tRNA₂^{Gln}(G1 A36 A38); K1, K2, K3, variants of tRNA₂^{Gln}(G1 A36 A38) bearing mutation at knob 1, 2 or 3 (see text); Val, acylated with valine; YPhe Val, valine-acylated suppressor tRNA derived from yeast tRNA^{Phe}.

k_{cat}/K_m for this tRNA is 1700-fold lower than that of the wild-type tRNA [9]. A mutation of U→A at position 38 in tRNA₂^{Gln}(G1 A36) increased the suppression efficiency to 65% (Figure 2), again consistent with previous *in vivo* observations of 2–40-fold greater suppression efficiencies [13] resulting from this mutation. The introduction of the knob 1 mutation (G3–C70→C3–G70) into the suppressor tRNA₂^{Gln}(G1 A36 A38) resulted in a threefold decrease in suppression efficiency to 21%. A slightly larger decrease (fourfold) resulted from the mutation at knob 2 (G10–C25→C10–G25), but the introduction of the knob 3 mutation (C16→G16) had little effect on suppression efficiency (Figure 2). As expected, the combination of the knob 1 and knob 3 mutations resulted in a mutant tRNA with a suppression efficiency (22%) similar to that of the knob 1 mutant. Surprisingly, however, the knob 1 and knob 2 mutations in combination (21% suppression) did not lead to a further decrease in suppression efficiency relative to the individual knob 1 or knob 2 mutants. Significantly, the tRNA bearing mutations at knobs 2 and 3, and the tRNA containing all three knob mutations were unable to suppress the nonsense mutation at position 88; suppression efficiencies were 6% for both tRNAs, compared with 7% readthrough product for the reaction lacking tRNA (Figure 2).

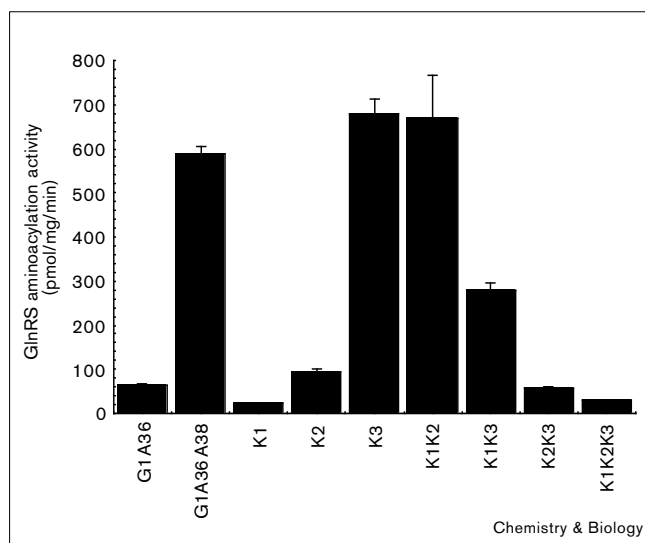
Suppression efficiencies of chemically acylated tRNAs

In order to determine whether low suppression efficiencies result from decreased aminoacylation levels or from the failure of the mutant tRNAs to be accepted by the translational elongation factor EF-Tu or the ribosome, the four tRNAs bearing combinations of two or three of the knob mutations were chemically aminoacylated and evaluated in the same *in vitro* protein synthesis assay. Runoff transcription of the *FokI*-digested templates provided the truncated tRNAs lacking the 3' CA dinucleotide. Enzymatic ligation of the truncated tRNAs to the chemically aminoacylated dinucleotide pdCpA-valine afforded full-length suppressor tRNAs acylated with valine [3], which were then added to the *in vitro* suppression reactions as described above. When tRNA bearing mutations at both knobs 1 and 2 was chemically acylated, full-length chorismate mutase was produced efficiently (56% suppression efficiency), suggesting that the low suppression efficiency of the unacylated tRNA is not the result of poor interactions with the translational machinery (Figure 2). In contrast, the chemically acylated tRNA with mutations at both knobs 1 and 3 suppressed the amber mutation with an efficiency (27%) comparable to that of the unacylated tRNA (Figure 2). This result is consistent with the partial failure of this tRNA to interact efficiently with the ribosome, EF-Tu or other translational factors. The chemically acylated tRNA bearing mutations at both knobs 2 and 3 again did not suppress the nonsense mutation (Figure 2), indicating a lack of translational competence and thus a lack of suitability as an orthogonal tRNA. The acylated tRNA bearing all three knob mutations, however, suppressed the amber codon in chorismate mutase with 39% efficiency; equal or greater amounts of full-length protein were produced with this tRNA than with the *supE* tRNA (Figure 2). This tRNA therefore satisfies the main requirements of an orthogonal tRNA — the inability to serve as a substrate for an endogenous aminoacyl-tRNA synthetase and the ability to function in translation.

Aminoacylation assays with *E. coli* glutamyl-tRNA synthetase

In order to measure aminoacylation activity directly, each of the mutant tRNAs was also assayed *in vitro* for its ability to be acylated by purified *E. coli* GlnRS at physiological concentrations of 3 mM ATP [14], 2 μM tRNA [15] and 150 μM glutamine [16]. Under these conditions, the suppressor tRNA₂^{Gln}(G1 A36) was aminoacylated at a rate 8900-fold slower than the wild-type tRNA₂^{Gln} (Figure 3). Introduction of the A38 mutation resulted in a substantial increase (ninefold) in aminoacylation rate by GlnRS (Figure 3). Addition of the knob 1 mutation to the suppressor tRNA₂^{Gln}(G1 A36 A38) resulted in a greater than 25-fold loss of GlnRS aminoacylation activity (Figure 3). This result is consistent with a 100-fold decrease in k_{cat}/K_m previously observed arising from a different mutation (A3–U70) at the same sites in tRNA₂^{Gln} [9]. Similarly,

Figure 3

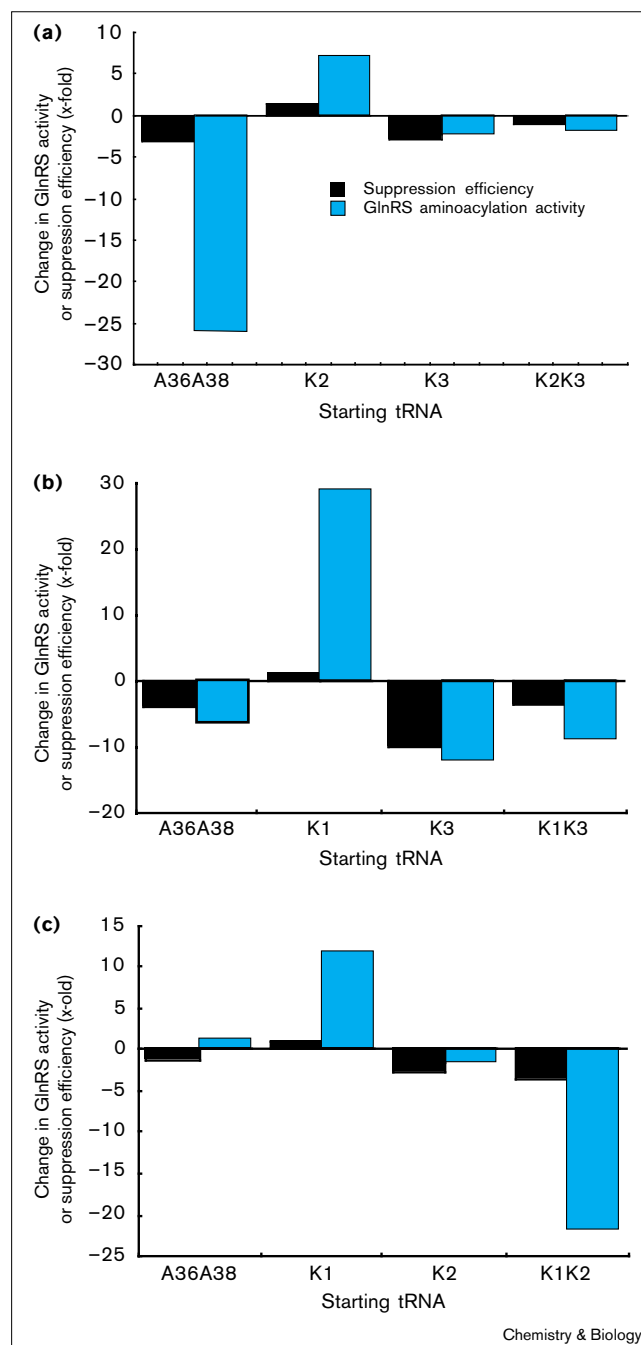


The GlnRS aminoacylation activities of mutant suppressor tRNAs. Assays (two to seven independent trials per tRNA) were conducted at 37°C under physiological concentrations of ATP, glutamine and tRNA (see text). Activities are reported in units of pmol glutamyl-tRNA produced per mg enzyme per min. Under these conditions, wild-type tRNA^{Gln} was acylated with a specific activity of 560,000 ± 68,000 pmol/mg/min. The identity of each tRNA is indicated using the same notation as in Figure 2.

the sixfold decrease in aminoacylation of the suppressor bearing the knob 2 mutation relative to that of tRNA₂^{Gln}(G1 A36 A38) parallels the 23-fold decrease in k_{cat}/K_m previously observed for the identical (C10-G25) changes introduced into tRNA₂^{Gln} [8]. Mutation of the tRNA₂^{Gln}(G1 A36 A38) suppressor at knob 3 did not result in a decrease in GlnRS aminoacylation activity (Figure 3), also consistent with previous findings that a different mutation at the same position (U16) in tRNA₂^{Gln} resulted in only a twofold decrease in k_{cat}/K_m [8].

When introduced in various combinations, mutations at knobs 1, 2 and 3 affect GlnRS aminoacylation in surprising ways. The aminoacylation activity of the suppressor tRNA with mutations at both knob 1 and knob 2 is similar to that of tRNA₂^{Gln}(G1 A36 A38), indicating that the negative effects of the individual knob 1 and knob 2 mutations on GlnRS aminoacylation cancel each other (Figures 3 and 4). Combination of the knob 3 mutation (which alone has little effect on aminoacylation activity [8]) with the knob 1 mutation resulted in a suppressor tRNA with an aminoacylation activity that was 12 times greater than that of the knob 1 mutant alone. In contrast, combination of the knob 3 mutation with the knob 2 mutation yielded a suppressor tRNA with an aminoacylation activity half that of the tRNA mutated at knob 2 (Figures 3 and 4). Importantly, GlnRS acylated

Figure 4



The effects of introducing each knob mutation on *in vitro* suppression efficiencies and GlnRS aminoacylation activities. (a) The effect of introducing knob 1. (b) The effect of introducing knob 2. (c) The effect of introducing knob 3. Black bars refer to changes in suppression efficiencies and blue bars refer to changes in GlnRS aminoacylation activities resulting from a given knob mutation. Increases in efficiency or activity are indicated by bars above the x axis and decreases are denoted by bars below the x axis.

the tRNA that was mutated at knobs 1, 2 and 3 19-fold slower than tRNA₂^{Gln}(G1 A36 A38) and 18,000-fold

slower than wild-type tRNA^{Gln}, confirming the inability of this tRNA to be charged by endogenous GlnRS.

***In vivo* suppression assays**

Given the occasional lack of agreement between *in vitro* and *in vivo* assays of nonsense suppression [17] and the complication of tRNA modification *in vivo*, we conducted a final experiment to confirm the suitability of the tRNA that was mutated at knobs 1, 2 and 3 to serve as the orthogonal tRNA *in vivo*. A genomic amber mutation in the *lacZ* gene of *E. coli* strain BT235 [18] renders BT235 unable to produce active β -galactosidase and therefore unable to grow on lactose as the sole carbon source. When transformed with pBRGlnS (expressing wild-type GlnRS) and with pACYCsupE (expressing the tRNA^{Gln}(A36) suppressor), BT235 double transformants survived on lactose minimal media at a rate approaching 100%. Transformation with pBRGlnS and with a pACYC derivative expressing the orthogonal suppressor tRNA bearing mutations at knobs 1, 2 and 3, however, allowed this strain to survive on lactose minimal media at a rate of only one in 100,000, indicating the inability of GlnRS to charge this tRNA with glutamine.

Discussion

Generation of an orthogonal tRNA

Guided by the X-ray crystal structure of the *E. coli* GlnRS–tRNA₂^{Gln} complex [6], we have generated several mutant tRNA₂^{Gln}-derived suppressors as candidates for an orthogonal tRNA capable of delivering unnatural amino acids site-specifically into proteins *in vivo*. Taken together, the *in vitro* and *in vivo* experiments confirm the suitability of the mutant tRNA₂^{Gln}(G1 C3 C10 G16 G25 A36 A38 G70) to serve as the orthogonal tRNA for the site-specific delivery of unnatural amino acids into proteins *in vivo* [5]. This tRNA is not a substrate for GlnRS and is not aminoacylated by any endogenous aminoacyl-tRNA synthetase in *E. coli*, yet possesses the structural features required for efficient translation. Efforts are underway to evolve mutant GlnRS enzymes capable of aminoacylating the orthogonal tRNA with natural and unnatural amino acids [5]. The characterization of mutant tRNAs that were generated during the course of these efforts evaluates the suitability of each tRNA to serve as the orthogonal suppressor and provides insights into the recognition of tRNA₂^{Gln} by its cognate aminoacyl-tRNA synthetase and the ribosome. A comparative analysis of the effects of the knob 1, knob 2 and knob 3 mutations on translational competence and enzymatic aminoacylation reveals several unexpected findings.

Properties of singly mutated suppressors

Earlier studies have suggested that increased suppression efficiencies of some A38-containing suppressors may arise from improved ribosomal binding of these tRNAs [11,19]. The ninefold increase in GlnRS aminoacylation activity

(Figure 3) resulting from the mutation of tRNA₂^{Gln}(G1 A36) to tRNA₂^{Gln}(G1 A36 A38) suggests that the enhanced *in vitro* and *in vivo* suppression efficiencies of the A38-containing tRNA [11,13] may also be accounted for, at least in part, by an increased rate of aminoacylation by GlnRS. The changes in GlnRS aminoacylation activity (Figure 3) and *in vitro* suppression efficiency (Figure 2) arising from introduction of each of the three knob mutations separately agree with previously reported changes arising from individual mutations at the same sites in wild-type tRNA₂^{Gln} [8–10,20]. This suggests that interactions of these positions in the tRNA with GlnRS are relatively independent of interactions involving bases 36 and 38 of the anticodon loop.

Properties of suppressors containing multiple sets of mutations

The effects of various combinations of the knob 1, knob 2 and knob 3 mutations on GlnRS aminoacylation activity differ from the effects of the individual mutations. Most noticeably, the knob 1 and knob 2 mutations separately confer 6–26-fold decreases in the ability of each suppressor to be aminoacylated by GlnRS, yet when combined afford a tRNA with a slightly greater GlnRS aminoacylation activity than tRNA₂^{Gln}(G1 A36 A38) (Figures 3 and 4). Similarly, the addition of the knob 3 mutation (which alone has little effect) to the tRNA bearing the knob 1 mutation increases the ability of the resulting suppressor to serve as a GlnRS substrate by more than 12-fold. At the same time, this mutation results in a 22-fold decrease in the GlnRS activity of the tRNA with mutations at knobs 1 and 2 (Figure 4).

Nonadditive effects arising from combination of these mutations were also observed in the translational efficiency of the suppressor tRNAs acylated with valine. The tRNA mutated at knobs 2 and 3 is no longer accepted by the protein biosynthetic machinery (Figure 2) but the addition of a third mutation (knob 1) remedies this defect. Similarly, the tRNA with mutations at knobs 1 and 3 is less efficient in translation than both the tRNAs bearing mutations at all three sites (Figure 2). These observations are surprising given that none of the mutated positions are invariant among *E. coli* tRNAs [21] and therefore a variety of bases at these positions are presented to the translational machinery.

The *in vitro* suppression efficiencies of the unacylated tRNAs reflect both the ability of the suppressors to be acylated by any endogenous *E. coli* aminoacyl-tRNA synthetase and the ability of these tRNAs to function in translation. Although the combination of these (and possibly other) factors complicates the interpretation of these suppression efficiencies, the above results again suggest that the knob 1, 2 or 3 mutations interact in a complex fashion. The lower suppression efficiencies of the individual

knob 1 and knob 2 mutants than that of tRNA₂^{Gln}(G1 A36 A38), for example, are not additive in the tRNA that is mutated at both knob 1 and knob 2 (Figure 2). Similarly, the addition of the knob 3 mutation to the suppressor tRNA bearing mutations at knobs 1 and 2 decreases suppression efficiency to a greater degree (fourfold) than the decrease (1.1-fold) arising from introduction of the knob 3 mutation to tRNA₂^{Gln}(G1 A36 A38) (Figure 2).

Comparison of the *in vitro* suppression data with the *in vitro* GlnRS aminoacylation data raises a number of interesting issues. For example, the level of suppression associated with the tRNA bearing the knob 1 mutation (Figure 2) is significantly higher than would be predicted on the basis of the inability of this tRNA to be aminoacylated by GlnRS (Figure 3). Indeed, the suppressor bearing all three knob mutations is aminoacylated slightly faster than the tRNA that is mutated only at knob 1 (Figure 3), yet is unable to suppress the amber codon *in vitro*. An obvious explanation is that tRNA aminoacylation is not rate determining in some *in vitro* suppression reactions. Alternatively, the suppressor bearing the mutation at knob 1, a site known to serve as an recognition element for many aminoacyl-tRNA synthetases [17,22–24], may be charged by a synthetase other than GlnRS during the *in vitro* translation reaction. The GlnRS aminoacylation activity of the tRNA mutated at knobs 1 and 2 also fails to correlate with the *in vitro* suppression behavior. Although this tRNA is aminoacylated by GlnRS at a similar rate to that of tRNA₂^{Gln}(G1 A36 A38) or the tRNA that is mutated at knob 3, it demonstrates a threefold lower suppression efficiency than either of these tRNAs despite its translational competence (Figures 2 and 3). Although difficult to explain, this inconsistency could arise from the deacylation of tRNA that is mutated at knobs 1 and 2 by another aminoacyl-tRNA synthetase.

Together, the effects of various combinations of mutations at knobs 1, 2 and 3 on GlnRS acylation activity, on the translational efficiency of these tRNAs and on *in vitro* suppression efficiency indicate that these three sets of mutations interact strongly and often in a nonadditive fashion (Figure 4). Given the relatively conservative nature of the mutations, it is unlikely that these changes significantly affect the overall structure of the tRNA. Although this degree of interaction is surprising given the distance between the three knob sites in the X-ray crystal structure of the GlnRS–tRNA₂^{Gln} complex (23–31 Å), it is consistent with previous findings that mutations at one location in GlnRS or in tRNA₂^{Gln} are often communicated to distant regions in GlnRS [7,20,25,26]. The correlation between mutations in different domains in GlnRS, and possibly in components of the ribosome, may help to explain the exquisite specificity of the protein biosynthetic machinery. More detailed explanations of these interactions will probably

require the structural characterization of these mutant tRNAs as complexes with GlnRS.

Significance

Guided by previously reported structural and biochemical properties of *Escherichia coli* glutamyl-tRNA synthetase (GlnRS) and tRNA₂^{Gln}, we have introduced mutations at three sites within tRNA₂^{Gln} to generate an 'orthogonal' tRNA suitable for the site-specific incorporation of unnatural amino acids into proteins *in vivo*. This tRNA is not a substrate for any *E. coli* aminoacyl-tRNA synthetase, yet functions efficiently during translation. *In vitro* suppression and aminoacylation assays have revealed the effects of these mutations, separately and in combination, on the ability of the resulting tRNAs to be acylated by *E. coli* aminoacyl-tRNA synthetases and to function in translation. Although the mutations are separated by 23–31 Å in the GlnRS–tRNA₂^{Gln} structure, the effects of mutations were found to correlate strongly. This correlation within different domains of aminoacyl-tRNA synthetases and the ribosome may contribute to the high fidelity of protein biosynthesis.

Materials and methods

Construction of tRNA genes

Genes encoding tRNAs for runoff transcription by T7 RNA polymerase were constructed from two overlapping synthetic oligonucleotides (Genosys) by primer extension using the Klenow fragment of DNA polymerase I. The resulting double-stranded construct contained, in order, a *KpnI* restriction site, the T7 promoter, the tRNA sequence, sites for *Bst*NI and *FokI* digestion, and a *Hind*III site. This construct was inserted between the *KpnI* and *Hind*III sites of pYPhe2 [27]. Digestion of the resulting plasmid with *Bst*NI or *FokI* resulted in templates suitable for transcription of full-length tRNA or truncated (–CA) tRNA, respectively. Genes encoding tRNAs for *in vivo* expression were similarly constructed from two overlapping synthetic oligonucleotides and inserted between the *Eco*RI and *Pst*I sites of pACYCsupE, placing transcription under control of the *lpp* promoter and the *rnc*C terminator. The sequences of the overlapping oligonucleotides used to construct the wild-type suppressor tRNA₂^{Gln}(A36) gene for runoff transcription are as follows with the tRNA sequence in italics: 5'-GCGGGG-TACCGCTCGAGTAATACGACTCACTATAGGGGGTATCGCCAAG-CGGTAAGGCACCGGATTCATTAATCCGGC-3'; 5'-GCGCGCAAGC TTGGATGGATCACCTGGCTGGGGTACGAGGATTCGAACCTCG-GAATGCCGGATTAGAATCCGG-3'. Oligonucleotides used to construct variants of this tRNA had mutations at the appropriate positions.

Runoff transcription and *in vitro* translation assays of suppressor tRNAs

Runoff transcription of the tRNAs was carried out as described [28]. The purity of the resulting tRNAs was analyzed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea. This procedure consistently yielded 2 mg of tRNA of greater than 80% purity per 0.5 ml runoff transcription reaction (the major contaminant was the n+1 product). *In vitro* transcription and translation reactions were performed as described previously [28] using 3 µg of plasmid containing the *E. coli* chorismate mutase gene bearing an amber mutation at site Gln88 and 10 µg of suppressor tRNA per 30 µl reaction at a final magnesium concentration of 7 mM. Reactions to detect acylation by endogenous *E. coli* aminoacyl-tRNA synthetases used full-length tRNA generated from the runoff transcription of *Bst*NI-digested template DNA described above; reactions to assay ribosomal acceptance used truncated tRNA generated from the runoff transcription of *FokI*-digested

DNA. This truncated tRNA was ligated using T4 RNA ligase to the dinucleotide pdCpA, which had been acylated with NVOC-protected valine, and then photodeprotected prior to addition to the transcription and translation reaction as described [3]. The resulting ³⁵S-Met-labeled crude reaction mixtures were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the amounts of truncated and full-length proteins were quantified using a Molecular Dynamics 445SI phosphorimager. Suppression efficiencies were defined as the amount of full-length protein divided by the sum of the full-length and truncated products.

GlnRS aminoacylation assays

Wild-type *E. coli* GlnRS was purified as described previously [29]. Specific activities were determined at concentrations corresponding to intracellular levels of 3 mM ATP [14], 150 μM glutamine [15] and 2 μM tRNA [16]. Assays (20 μl total volume) were carried out at 37°C and contained 40 mM Hepes (pH 7.2), 10 mM MgCl₂, 50 mM KCl, 2 mM β-mercaptoethanol, ATP, glutamine (1–100 mol% radiolabeled with ³H or ¹⁴C, Dupont NEN), tRNA and GlnRS. Assays conducted at 30°C provided similar results (data not shown). Enzyme concentrations were adjusted such that less than 10% of limiting substrate (tRNA) was consumed. Reactions were stopped after 60 s by pipetting onto a Whatman 3MM filter disc pretreated with 10% trichloroacetic acid (TCA), washed once in 10% TCA, and washed four to five times in 5% TCA for 10 min per wash. Filters were then rinsed once with ethanol, rinsed three times with diethyl ether, dried under a stream of air, and counted by scintillation. Wild-type *E. coli* tRNA was used as a mix of all tRNAs from *E. coli* (Boehringer Mannheim); tRNA^{Gln} constitutes 1.8% of whole *E. coli* tRNA [30].

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References

- Cornish, V.W., Mendel, D. & Schultz, P.G. (1995). Probing protein structure and function with an expanded genetic code. *Angew. Chem. Int. Ed. Engl.* **34**, 621-633.
- Mendel, D., Cornish, V.W. & Schultz, P.G. (1995). Site-directed mutagenesis with an expanded genetic code. *Annu. Rev. Biophys. Biomol. Struct.* **24**, 435-462.
- Thorson, J.S., Cornish, V.W., Barrett, J.E., Cload, S.T., Yano, T. & Schultz, P.G. (1997). A biosynthetic approach for the incorporation of unnatural amino acids into proteins. *Methods in Mol. Biol.: Protein Synthesis: Methods and Protocols*, in press.
- Nowak, N.W., *et al.*, & Lester, H.A. (1995). Nicotinic receptor binding site probed with unnatural amino acid incorporation in intact cells. *Science* **268**, 439-442.
- Liu, D.R., Magliery, T.J., Pasternak, M. & Schultz, P.G. (1997). Engineering a tRNA and aminoacyl-tRNA synthetase for the site-specific incorporation of unnatural amino acids into proteins *in vivo*. *Proc. Natl Acad. Sci. USA*, in press.
- Rould, M.A., Perona, J.J. & Steitz, T.A. (1991). Structural basis of anticodon loop recognition by glutamyl-tRNA synthetase. *Nature* **352**, 213-218.
- Sherman, J.M., Thomann, H. & Söll, D. (1996). Functional connectivity between tRNA binding domains in glutamyl-tRNA synthetase. *J. Mol. Biol.* **256**, 818-828.
- Hayase, Y., *et al.*, & Söll, D. (1992). Recognition of bases in *Escherichia coli* tRNA^{Gln} by glutamyl-tRNA synthetase: a complete identity set. *EMBO J.* **11**, 4159-4165.
- Jahn, M., Rogers, M.J. & Söll, D. (1991). Anticodon and acceptor stem nucleotides in tRNA^{Gln} are major recognition elements for *E. coli* glutamyl-tRNA synthetase. *Nature* **352**, 258-260.
- Englisch-Peters, S., Conley, J., Plumbridge, J., Leptak, C., Söll, D. & Rogers, M.J. (1991). Mutant enzymes and tRNAs as probes of the glutamyl-tRNA synthetase: tRNA^{Gln} interaction. *Biochimie* **73**, 1501-1508.
- Bradley, D., Park, J.V. & Soll, L. (1981). tRNA^{Gln} Su+2 mutants that increase amber suppression. *J. Bacteriol.* **145**, 704-712.
- Stewart, J., Wilson, D.B. & Ganem, B. (1990). A genetically engineered monofunctional chorismate mutase. *J. Am. Chem. Soc.* **112**, 4582-4584.
- Kleina, L.G., Masson, J.M., Normanly, J., Abelson, J. & Miller, J.H. (1990). Construction of *Escherichia coli* amber suppressor tRNA genes. II. Synthesis of additional tRNA genes and improvement of suppressor efficiency. *J. Mol. Biol.* **213**, 705-717.
- Neidhardt, F. & Umberger, H.E. (1996). In *Escherichia coli and Salmonella*. (Neidhardt, F., ed.) pp. 13-16, ASM Press, Washington, D.C.
- Ikemura, T. (1985). Codon usage and tRNA content in unicellular and multicellular organisms. *Mol. Biol. Evol.* **2**, 13-34.
- Raunio, R. & Rosenqvist, H. (1970). Amino acid pool of *Escherichia coli* during the different phases of growth. *Acta. Chim. Scand.* **24**, 2737-2744.
- McClain, W.H. (1993). Rules that govern tRNA identity in protein synthesis. *J. Mol. Biol.* **234**, 257-280.
- Yamao, F., Inokuchi, H. & Ozeki, H. (1988). Mischarging mutants of Su+2 glutamine tRNA in *E. Coli*. *Jpn. J. Genet.* **63**, 237-249.
- Geffer, M.L. & Russell, R.L. (1969). Role of modifications in tyrosine transfer RNA: a modified base affecting ribosome binding. *J. Mol. Biol.* **39**, 145-147.
- Ibba, M., Hong, K.-W., Sherman, J.M., Sever, S. & Söll, D. (1996). Interactions between tRNA identity nucleotides and their recognition sites in glutamyl-tRNA synthetase determine the cognate amino acid affinity of the enzyme. *Proc. Natl Acad. Sci. USA* **93**, 6953-6958.
- RajBhandary, U.L. & Chow, C.M. (1995). In *tRNA: Structure, Biosynthesis, and Function*. (Söll, D. & RajBhandary, U.L., eds) p. 97, ASM Press, Washington.
- RajBhandary, U.L. & Chow, C.M. (1995). In *tRNA: Structure, Biosynthesis, and Function*. (Söll, D. & RajBhandary, U.L., eds) p. 389, ASM Press, Washington.
- Schulman, L.H. (1991). Recognition of tRNAs by aminoacyl-tRNA synthetases. *Prog. Nucleic Acid Res.* **41**, 23-87.
- Normanly, J. & Abelson, J. (1989). tRNA identity. *Annu. Rev. Biochem.* **58**, 1029-1049.
- Rogers, M.J., Adachi, T., Inokuchi, H. & Söll, D. (1994). Functional communication in the recognition of tRNA by *Escherichia coli* glutamyl-tRNA synthetase. *Proc. Natl Acad. Sci. USA* **91**, 291-295.
- Weygand-Durasevic, I., Rogers, M.J. & Söll, D. (1994). Connecting anticodon recognition with the active site of *Escherichia coli* glutamyl-tRNA synthetase. *J. Mol. Biol.* **240**, 111-118.
- Noren, C., Anthony-Cahill, S.J., Suich, D.J., Noren, K.A., Griffith, M.C. & Schultz, P.G. (1990). *In vitro* suppression of an amber mutation by a chemically aminoacylated transfer RNA prepared by runoff transcription. *Nucleic Acids Res.* **18**, 83-88.
- Cload, S.T., Liu, D.R., Froland, W.A. & Schultz, P.G. (1996). Development of improved tRNAs for *in vitro* biosynthesis of proteins containing unnatural amino acids. *Chem. Biol.* **3**, 1033-1038.
- Thomann, H.-U., Ibba, M., Hong, K.-W. & Söll, D. (1996). Homologous expression and purification of mutants of an essential protein by reverse epitope-tagging. *Biotechnology* **14**, 50-55.
- Lloyd, A.J., Thomann, H.-U., Ibba, M. & Söll, D. (1995). A broadly applicable continuous spectrophotometric assay for measuring aminoacyl-tRNA synthetase activity. *Nucleic Acids Res.* **23**, 2886-2892.