Interactions between tRNA identity nucleotides and their recognition sites in glutaminyl-tRNA synthetase determine the cognate amino acid affinity of the enzyme

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ABSTRACT Sequence-specific interactions between aminoacyl-tRNA synthetases and their cognate tRNAs both ensure accurate RNA recognition and prevent the binding of noncognate substrates. Here we show for Escherichia coli glutaminyl-tRNA synthetase (GlnRS; EC 6.1.1.18) that the accuracy of RNA recognition also determines the efficiency of cognate amino acid recognition. Steady-state kinetics revealed that interactions between tRNA identity nucleotides and their recognition sites in the enzyme modulate the amino acid affinity of GlnRS. Perturbation of any of the protein–RNA interactions through mutation of either component led to considerable changes in glutamine affinity with the most marked effects seen at the discriminator base, the 10:25 base pair, and the anticodon. Reexamination of the identity set of tRNAGln in the light of these results indicates that its constituents can be differentiated based upon biochemical function and their contribution to the apparent Gibbs' free energy of tRNA binding. Interactions with the acceptor stem act as strong determinants of tRNA specificity, with the discriminator base positioning the 3' end. The 10:25 base pair and U35 are apparently the major binding sites to GlnRS, with G36 contributing both to binding and recognition. Furthermore, we show that E. coli tryptophanyl-tRNA synthetase also displays tRNA-dependent changes in tryptophan affinity when charging a noncognate tRNA. The ability of tRNA to optimize amino acid recognition reveals a novel mechanism for maintaining translational fidelity and also provides a strong basis for the coevolution of tRNAs and their cognate synthetases.

The aminoacyl-tRNA synthetases (AARSs; ref. 1) are a ubiquitous family of enzymes whose primary function is to provide the cell with correctly paired tRNAs and amino acids for mRNA translation. The fundamental requirement of this reaction to the accurate flow of genetic information is reflected by the extreme age (2) and specificity (3) of this group of enzymes. The specificity of AARSs, which is essential to reduce translational errors to a tolerable level (4), is achieved through stringent discrimination against both noncognate amino acids (5) and tRNAs (6, 7) and competition between cognate and noncognate substrates (8). tRNA selection is based on the existence of mutually exclusive interactions between certain bases in a tRNA (the "identity set"; ref. 9) and recognition sites in its cognate AARS, whereas most amino acids can be readily distinguished by differences in their physicochemical properties. Although this process of selection is generally highly accurate, it does not allow for absolute discrimination against noncognate substrates (10). For example, the amino acids valine and isoleucine are sufficiently similar in structure to allow their recognition by both valyl- and isoleucyl-tRNA synthetase, but the propagation of translational errors as a result of such mistakes is prevented by proofreading of the noncognate intermediate or product (11, 12). Some AARSs are also able to aminoacylate a variety of tRNAs despite significant differences in their nucleotide sequences (13), but in contrast to misacylated amino acids these are not subsequently proofread. Perhaps the most promiscuous AARS in this respect is Escherichia coli glutaminyl-tRNA synthetase (GlnRS), which charges mutants of the amber suppressor tRNA<sup>tyr</sup>(<i>tyr</i>(UAG))<i>in vivo</i> (14, 15). Surprisingly, even when GlnRS is mutated to enhance significantly mischarging of both this and other tRNA acceptor species, little or no change in cell viability is observed. This is explained by the recent observation of glutamine recognition in GlnRS in tRNAs dependent (16). The complexes noncognate tRNAs form with GlnRS are significantly less efficient at glutamine recognition and aminoacylation than cognate complexes, which favors the formation of correctly paired products. In its function, tRNA-dependent cognate amino acid recognition is therefore comparable to a proofreading mechanism because it acts to reduce the error rate of GlnRS.

The detection of tRNA-dependent amino acid recognition suggests that nucleotide identity sets may determine not only the affinity of a tRNA for its cognate AARS but also the efficiency of the subsequent aminoacylation reaction. GlnRS: tRNA<sup>Gln</sup> of E. coli presents an excellent model system in which to further investigate this hypothesis. The crystal structure of the ternary complex GlnRS:tRNA<sup>Gln</sup>:ATP has been solved at high resolution (17, 18) and the recognition elements of both components have been studied extensively (19–23). We therefore set out to investigate the effects of mutations in the tRNA<sup>Gln</sup> identity set and GlnRS recognition sites on the steady-state kinetics of glutamine recognition and aminoacylation. Because it has previously been shown that certain anticodon variants of tRNA<sup>Gln</sup> are also efficiently charged with tryptophan (24), we investigated the effect of these particular mutations on the cognate amino acid affinity of tryptophanyl-tRNA synthetase (TrpRS). Such data would provide insights into both the role of tRNA identity elements during recognition by a noncognate AARS and the generality of tRNA-dependent cognate amino acid recognition. The second point is of particular interest since GlnRS, unlike TrpRS, falls into a small subgroup of AARSs (with glutamyl- and arginyl-tRNA synthetase), which require the presence of tRNA for aminoacyl–adenylate formation (25). The possible effects of tRNA-dependent amino acid recognition on the kinetics of the third substrate of the aminoacylation reaction, ATP, were not investigated as its use is common to all the aminoacyl-tRNA synthetases.

MATERIALS AND METHODS


Abbreviations: AARS, aminoacyl-tRNA synthetase; GlnRS, glutamyl-tRNA synthetase; TrpRS, tryptophanyl-tRNA synthetase.

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proteins has been described. Mutants of a tRNA^{Gln} gene containing G at position 1 were prepared by in vitro transcription and purification as described (19, 20, 24). TrpRS and in vitro transcribed tRNA\textsuperscript{Trp}\textsubscript{ΔA1} were prepared as recently detailed (26), in vivo E. coli tRNA\textsuperscript{Trp} was purchased from Subrider RNA (Rolling Bay, WA). Media for bacterial growth and molecular biology protocols were standard unless otherwise noted (27).

**Strains.** The E. coli strain BL21(DE3) (28) was used for the overexpression of wild-type GlnRS. GlnRS-R402A was overexpressed in strain HAPPY101 (29) and the variants K317R and Q318K were overexpressed in the glnS deletion strain X3R2 (30).

**Plasmids.** Wild-type GlnRS was inducibly overexpressed from plasmid pET33glnSwt (31). GlnRS variants K317R, Q318K, and R402A were overexpressed as pBR clones (16).

**Protein Purification.** Wild-type, K317R, Q318K, and R402A GlnRS were purified as described (29) except that immunodetection of HA-GlnRS was omitted for the first three.

**Aminocacylation Assays.** All aminocacylation assays were performed at 37°C in a reaction mixture containing 20 mM Hepes (pH 7.2), 10 mM magnesium acetate, 5 mM ATP, and 5 μM-6.4 mM glutamine (45 Ci/mmol; 1 Ci = 37 GBq). The concentration of ATP used was 20-fold higher than its K\textsubscript{M} for wild-type GlnRS and 2- to 3-fold higher than for previously characterized ATP binding mutants of GlnRS (16). For the determination of kinetic parameters for glutamine with GlnRS mutants, in vivo generated tRNA\textsuperscript{Gln} was added at a final concentration of 2 μM for wild-type and D235N; 5 μM for L136A, L136F, K317R, Q318K, and E323G; 10 μM for R402A; and 30 μM for R341A. These concentrations were based on the previously determined K\textsubscript{M} for tRNA\textsuperscript{Gln} of these enzymes. GlnRS concentration was in the range of 1–20 nM in all cases. For the determination of kinetic parameters for glutamine with mutant tRNA\textsuperscript{Gln} G1 transscripts, the tRNA concentration was 4 μM for G1 itself; 6 μM for A73, A3; U70, G25, U16, and C10; 9 μM for C10:G25 and U36; 10 μM for U70, A35, and C35; and 16 μM for C73, U73, A2; U71, and A36. These transcript concentrations were based on their previously determined K\textsubscript{M} values (19, 20). The final GlnRS concentration was generally in the range of 10–80 nM, ensuring a molar ratio of at least 1:100 between the enzyme and all the substrates. The only exceptions were A35 and C35 where the final GlnRS concentration was 200 nM resulting in an initial ratio of 1:50 between GlnRS and tRNA (see Results for details). Aminocacylation reactions were processed as described (32) and kinetic parameters were calculated from Eadie–Hofstee plots. Aminocacylation by TrpRS was performed as described (24) except that the TrpRS concentration was 20 nM, ATP was 2 mM, and transcript concentrations were 10 μM for both tRNA\textsuperscript{Trp}\textsubscript{ΔA1} and tRNA\textsuperscript{Glu}\textsubscript{ΔCA} G1.

**RESULTS**

**Steady-State Glutaminylation of tRNA\textsuperscript{Gln}G1 in Vitro Transcription.** The steady-state kinetic parameters for glutamine binding (K\textsubscript{M}) during aminocacylation and the turnover number of the enzyme (k\textsubscript{cat}) were investigated using in vitro transcribed variants of a tRNA\textsuperscript{Gln} gene containing G instead of U at position 1 (referred to below as tRNA\textsuperscript{Gln}G1). Pyrophosphohate exchange could not be used to determine steady-state kinetic parameters for Gln-AMP formation because the reaction is completely tRNA dependent (33). The G1 mutation alone increased the k\textsubscript{cat} compared with in vivo produced tRNA\textsuperscript{Gln} but had little effect on the K\textsubscript{M} for glutamine (Table 1). This is in agreement with the structural data for this transcript complexed with GlnRS, which showed no differences when compared with the complex containing in vivo made tRNA\textsuperscript{Gln} (34). The observed increase in k\textsubscript{cat} is consistent with the proposed role of certain modifications in stabilizing the tRNA\textsuperscript{Gln}; GlnRS complex (34). Reducing the stability of the complex would be expected to facilitate substrate turnover resulting in a higher k\textsubscript{cat} as previously observed for the charging of tRNA\textsuperscript{Phe} with noncognate amino acids by phenylalanyl-tRNA synthetase (35). These relatively minor changes in the kinetic parameters indicated that tRNA\textsuperscript{Gln}G1 provided a suitable framework within which to probe the effect of mutations in tRNA\textsuperscript{Gln} identity elements (Fig. 1) on glutamine binding (Table 1).

**The Identity Nucleotides of tRNA\textsuperscript{Gln} Determine Glutaminylation Efficiency.** Mutation of nucleotides in the acceptor stem previously implicated in tRNA identity showed diverse effects on kinetic parameters for glutamine. All alterations of the discriminator base G73 led to substantial increases in the K\textsubscript{M} for glutamine and also resulted in less significant changes

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**Table 1.** GlnRS aminocacylation kinetics of tRNA\textsuperscript{Gln} mutants

<table>
<thead>
<tr>
<th>tRNA\textsuperscript{Gln}</th>
<th>k\textsubscript{cat}, min\textsuperscript{-1}</th>
<th>K\textsubscript{M}, μM Gln</th>
<th>k\textsubscript{cat}/K\textsubscript{M}, min\textsuperscript{-1}μM\textsuperscript{-1}</th>
<th>Fold decrease in k\textsubscript{cat}/K\textsubscript{M}</th>
<th>Aminocacylation performed at 37°C in standard buffer with Gln levels varied over the range of approximately 0.2–4 times K\textsubscript{M} with all other substrates at saturating concentrations.</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>168 ± 15</td>
<td>214 ± 37</td>
<td>0.79</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>In vitro (G1)</td>
<td>298 ± 17</td>
<td>289 ± 43</td>
<td>1.03</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Discriminator base</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-A73</td>
<td>762 ± 43</td>
<td>1610 ± 148</td>
<td>0.47</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>-C73</td>
<td>109 ± 8</td>
<td>1560 ± 208</td>
<td>0.07</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>-U73</td>
<td>156 ± 8</td>
<td>1057 ± 112</td>
<td>0.15</td>
<td>6.7</td>
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</tr>
<tr>
<td>Acceptor stem</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-A2;U71</td>
<td>396 ± 14</td>
<td>262 ± 20</td>
<td>1.51</td>
<td>0.68</td>
<td></td>
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<tr>
<td>-A3;U70</td>
<td>362 ± 5</td>
<td>258 ± 33</td>
<td>1.4</td>
<td>0.74</td>
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<tr>
<td>-U70</td>
<td>200 ± 17</td>
<td>797 ± 126</td>
<td>0.25</td>
<td>4.2</td>
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<tr>
<td>D-arm</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>-C10</td>
<td>420 ± 37</td>
<td>305 ± 42</td>
<td>1.38</td>
<td>0.77</td>
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<tr>
<td>-G25</td>
<td>391 ± 29</td>
<td>485 ± 32</td>
<td>0.81</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>-C10;G25</td>
<td>78 ± 6</td>
<td>1351 ± 150</td>
<td>0.06</td>
<td>17</td>
<td></td>
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<tr>
<td>Anticonodon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-A35</td>
<td>9.1 ± 1.3</td>
<td>5620 ± 990</td>
<td>1.62 × 10\textsuperscript{-3}</td>
<td>640</td>
<td></td>
</tr>
<tr>
<td>-C35</td>
<td>14 ± 0.3</td>
<td>4880 ± 122</td>
<td>2.95 × 10\textsuperscript{-3}</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>-A36</td>
<td>46 ± 6</td>
<td>1818 ± 348</td>
<td>0.025</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>-U36</td>
<td>373 ± 28</td>
<td>1117 ± 126</td>
<td>0.33</td>
<td>3</td>
<td></td>
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</tbody>
</table>

*Compared to in vitro transcribed G1 transcript.

\textsuperscript{f}Approximate values as these experiments were performed at sub-K\textsubscript{M} concentrations of tRNA.
This suggests that the identity of the nucleotides at positions 10 and 25 rather than the structural integrity of the D-arm itself is important for efficient glutaminylation. Whether the changes seen for the C10-G25 double mutant result solely from the direct disruption of specific interactions or also reflect indirect perturbation of interactions elsewhere in the GlnRS-tRNA\textsuperscript{Gln} complex is not clear.

The most striking changes in glutaminylation were observed for mutation of the anticodon nucleotides U35 and G36. The introduction of either A or C at position 35 led to a 20-fold increase in the \(K_M\) for glutamine and a 20- to 30-fold decrease in \(k_{cat}\), indicating that U35 is essential for efficient glutaminylation. Mutation of G36 to A also led to significant changes in both the \(k_{cat}\) and \(K_M\) for glutamine, although the resulting 40-fold decrease in catalytic efficiency was 10 times less than observed for U35 substitutions. U36 showed the least pronounced effect of all the anticodon mutations with a 4-fold increase in \(K_M\) and a 5-fold fall in catalytic efficiency.

An interesting feature of all the tRNA\textsuperscript{Gln} transcripts is that they generally show at least an order of magnitude higher \(k_{cat}\) values than previously reported (19, 20). This is readily explained by the glutamine concentration previously employed, 100 \(\mu\)M, which we have now shown to be sub-saturating in all cases, resulting in significant reduction of the observed rate of product formation and, consequently, \(k_{cat}\). This conclusion is supported by the recent observation that increasing the glutamine concentration to 400 \(\mu\)M led to substantially higher \(k_{cat}\) values than originally observed for anticodon mutants of tRNA\textsuperscript{Gln} G1 (30).

### Glutaminylation Kinetics of tRNA Recognition-Site Mutants of GlnRS

The steady-state kinetic parameters for glutamine were determined for a number of GlnRS variants mutated at positions known to interact with nucleotides in tRNA\textsuperscript{Gln} (Table 2). Substitution of residues L136 and D235, which interact with the identity elements U1:A72 and both G2:C71 and G3:C70 respectively, led to changes in the kinetic parameters of glutaminylation. The most significant differences were observed for L136A, which showed a 5-fold increase, and D235N, which showed a 40-fold decrease, with respect to the \(K_M\) for glutamine. However, both these and the other acceptor stem-binding mutants investigated, with the exception of L136F, showed little overall change in the catalytic efficiency of glutaminylation. By contrast, mutation of K317 and Q318, both of which make contacts with the inside of the L-shaped tRNA but not with known identity elements, led to a 4- to 6-fold decrease in the \(K_M\) for glutamine and a 1.5- to 2-fold increase in \(k_{cat}\) resulting in a significant improvement in the catalytic efficiency in both cases. Thus the disruption of

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**Table 2. Aminoacylation kinetics of GlnRS mutants**

<table>
<thead>
<tr>
<th>GlnRS recognition site</th>
<th>(k_{cat}, \text{min}^{-1})</th>
<th>(K_M, \mu\text{M})</th>
<th>(k_{cat}/K_M, \text{min}^{-1}\mu\text{M}^{-1})</th>
<th>Fold decrease in (k_{cat}/K_M*)</th>
<th>(\Delta AG\text{,kRNA}^{\text{Gln}})</th>
<th>(\text{kcal mol}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L136A</td>
<td>168 ± 15</td>
<td>214 ± 37</td>
<td>0.79</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L136F</td>
<td>472 ± 41</td>
<td>987 ± 101</td>
<td>0.48</td>
<td>1.6</td>
<td>—0.03</td>
<td>—</td>
</tr>
<tr>
<td>D235H*</td>
<td>48 ± 9</td>
<td>219 ± 17</td>
<td>0.22</td>
<td>3.6</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>D235N</td>
<td>119 ± 7</td>
<td>223 ± 24</td>
<td>0.53</td>
<td>1.5</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>K317R</td>
<td>41 ± 0.6</td>
<td>37 ± 2</td>
<td>1.1</td>
<td>0.72</td>
<td>—0.7</td>
<td>—</td>
</tr>
<tr>
<td>Q318K</td>
<td>387 ± 19</td>
<td>52 ± 6</td>
<td>7.4</td>
<td>0.1</td>
<td>—0.1</td>
<td>—</td>
</tr>
<tr>
<td>E323G</td>
<td>268 ± 17</td>
<td>38 ± 7</td>
<td>7.1</td>
<td>0.1</td>
<td>0.2</td>
<td>—</td>
</tr>
<tr>
<td>R341A</td>
<td>11 ± 2</td>
<td>691 ± 132</td>
<td>0.016</td>
<td>50</td>
<td>—2.6</td>
<td>—</td>
</tr>
<tr>
<td>R402A</td>
<td>176 ± 28</td>
<td>871 ± 170</td>
<td>0.2</td>
<td>4</td>
<td>—2.5</td>
<td>—</td>
</tr>
<tr>
<td>G36</td>
<td>372 ± 24</td>
<td>131 ± 15</td>
<td>2.8</td>
<td>0.3</td>
<td>—1.2</td>
<td>—</td>
</tr>
</tbody>
</table>

Aminoacylation experiments were performed at 37°C in standard buffer with Gln levels varied over the range of approximately 0.2–5 times \(K_M\) with all other substrates at saturating concentrations. ND, not determined.

*Compared to wild-type GlnRS.

\*Apparent difference between mutant and wild type in the Gibbs’ free energy for tRNA\textsuperscript{Gln} binding. \(\Delta AG = RT \ln \left( \frac{k_{cat}/K_M\text{mut}}{k_{cat}/K_M\text{wild}} \right)\). Values were calculated using the appropriate previously determined kinetic parameters for tRNA\textsuperscript{Gln} (see refs. 21, 22, and 30).

\*Data taken from Hong et al. (16).
certain interactions between GlnRS and both the acceptor stem and the inside of the folded L-shaped tRNA\textsuperscript{Gln} lead to significant changes in the efficiency of glutamine recognition. Because K317R and Q318K have previously been shown to display relaxed specificity of anticodon recognition (30), these results also suggest that a compromise exists between the stringency of anticodon recognition and the efficiency of glutamine recognition and subsequent aminoclaylation.

Changes in the efficiency of glutaminylation were also observed after mutation of the residues involved in recognition of the G10:C25 and U35 identity elements of tRNA\textsuperscript{Gln}. Replacement of E323, which interacts with the G10:C25 base pair in the D-arm, led to both a significant reduction in $k_{\text{cat}}$ (15-fold) and an increase in $K_M$ (3-fold) compared with wild-type confirming the data described above for the complementary mutation (C10:G25) in tRNA\textsuperscript{Gln} G1. Removal of the interaction between the anticodon nucleotide U35 and R341 by means of an alanine substitution at this residue caused a 4-fold increase in the $K_M$ for glutamine but had no effect on $k_{\text{cat}}$. In contrast, the only significant effect of removing the interaction between G36 and R402 was to double the $k_{\text{cat}}$. These effects are less pronounced than for mutations of the corresponding nucleotides in tRNA\textsuperscript{Gln} G1, indicating that the nucleotide replacements are considerably more disruptive to the integrity of the tRNA\textsuperscript{Gln}:GlnRS complex.

Aminoclaylation of tRNA\textsuperscript{Gln} Anticodon Mutants by TrpRS.

The steady-state kinetic parameters for tryptophan of wild-type TrpRS were determined in the presence of either the tRNA\textsuperscript{Gln}ΔA1 or tRNA\textsuperscript{Gln} G1 transcripts (Table 3). Aminoclaylation of the tRNA\textsuperscript{Gln}-derived transcript led to a 3-fold increase in the $K_M$ for tryptophan and a 3-fold decrease in $k_{\text{cat}}$ when compared with the kinetic parameters determined in the presence of tRNA\textsuperscript{Gln}ΔA1. In addition, the $K_M$ for tryptophan was a further 2-fold lower in the presence of in vivo produced tRNA\textsuperscript{Trp}. These results indicate that the cognate amino acid affinity of TrpRS is tRNA dependent.

**DISCUSSION**

Refining the Definition of the Identity Set of tRNA\textsuperscript{Gln}.

Although the identity sets of numerous tRNAs have been characterized, almost no data exist concerning whether, as earlier predicted (38), the constituents of such sets can be differentiated based upon their biochemical function. The only exception to this rule is the discriminator base, which has been shown to play a clearly defined role in orienting the 3′ terminus (39, 40). Following the earlier detection of tRNA-dependent cognate amino acid recognition by GlnRS, we have now reexamed the identity elements of tRNA\textsuperscript{Gln} with respect to the steady-state kinetics of glutaminylation. This showed that our previous experiments had been conducted at significantly sub-saturating conditions for glutamine, leading to substantial underestimations of $k_{\text{cat}}$ in several cases. When we now consider these changes in conjunction with the data presented both here and elsewhere for the mutation of tRNA recognition elements in GlnRS, it is clear that the identity set can in fact be subdivided based on two distinct biochemical functions: substrate recognition and binding (data for the discriminator base are in agreement with the function described above). It should be noted that these two activities would not be expected to function completely independently as the domains of GlnRS display a high degree of functional connectivity (reviewed in ref. 41). A more accurate delineation of the roles of various nucleotides requires a pre-steady-state analysis of glutaminylation, which will allow the effects of particular mutations to be assigned to certain steps of the reaction.

Specificity determinants of tRNA\textsuperscript{Gln}. The interactions between the discriminator base, the acceptor stem, and the inside of the L-shaped tRNA with GlnRS were all shown to contribute significantly to the $K_M$ for glutamine during aminoclaylation, in agreement with the earlier proposal that the orientation of this region optimizes the active site for cognate amino acid recognition. When the $k_{\text{cat}}$ values from these experiments (Tables 1 and 2) are considered in conjunction with the appropriate $K_{\text{cat}}$ (refs. 19–22 and 30 and L. Silvian and T. A. Steitz, personal communication) only small changes are observed in the efficiency of aminoclaylation upon mutation of either the acceptor stem itself or its recognition sites. However, disruption of any of these interactions through appropriate amino acid substitutions in GlnRS leads to significant reductions in tRNA specificity, as shown by the expanded range of amber suppressor tRNAs glutaminyalted in vivo (21–23). This suggests that the role of this region is to ensure the accuracy of substrate recognition by means of a network of individually weak interactions. A similar pattern was also observed following saturation mutagenesis of residues 126–138 in GlnRS, which make a number of contacts with the acceptor stem (31). This proposed role for the acceptor stem is supported by the low level of conservation between prokaryotes and eukaryotes of either this region of the tRNA (Fig. 2), or the corresponding positions in GlnRS (42) since mutation of either partner would not significantly change the efficiency of complex formation.

**tRNA-binding elements.** Calculation of the apparent Gibbs's free energy for tRNA\textsuperscript{Gln} binding by the various GlnRS mutants suggests that the major binding interactions occur with the 10:25 base pair and the anticodon (Table 2). Disruption of the interaction between either the 10:25 base pair or U35 and GlnRS led to large reductions in the efficiency of aminoclaylation (Tables 1 and 2), but did not result in any reduction in the in vivo specificity of tRNA recognition by GlnRS mutants (22). This is in direct contrast to the acceptor stem interactions, which are critical for tRNA recognition but contribute significantly less apparent binding energy to the GlnRS:tRNA\textsuperscript{Gln} complex (Table 2). The interaction between G36 and R402 plays a dual role because it contributes significantly both to tRNA binding and discrimination against noncognate tRNAs (22). The contribution of G10:C25, U35, G36, and the corresponding positions in GlnRS (G323, R341, and R402), to the stability of the protein–RNA interaction suggests that these nucleotides and amino acids should be well conserved. This was confirmed by the alignment of either tRNA\textsuperscript{Gln} genes from sources known to contain GlnRS activity (Fig. 2) or GlnRS

<table>
<thead>
<tr>
<th>tRNA</th>
<th>$k_{\text{cat}}$, min$^{-1}$</th>
<th>$K_M$, μM Trp</th>
<th>$k_{\text{cat}}/K_M$, min$^{-1}$μM$^{-1}$</th>
<th>Fold decrease in $k_{\text{cat}}/K_M^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>in vivo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA\textsuperscript{Trp}</td>
<td>288 ± 54</td>
<td>3.9 ± 0.1</td>
<td>74</td>
<td>1</td>
</tr>
<tr>
<td>in vitro</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA\textsuperscript{Gln}ΔA1</td>
<td>178 ± 2</td>
<td>6.8 ± 0.2</td>
<td>26</td>
<td>2.8</td>
</tr>
<tr>
<td>tRNA\textsuperscript{Gln} G1</td>
<td>52 ± 2</td>
<td>23 ± 1</td>
<td>2.2</td>
<td>34</td>
</tr>
</tbody>
</table>

**Table 3.** Aminoclaylation kinetics for TrpRS with Trp in the presence of cognate and noncognate tRNAs

Aminoclaylation experiments were performed at 37°C in standard buffer with Trp levels varied over the range of approximately 0.3–5 times $K_M$ with all other substrates at saturating concentrations.

*Compared to in vivo tRNA\textsuperscript{Trp}.
amino acid sequences (42), both of which show almost complete conservation of the relevant positions. This would be expected because mutation of any of these positions would be extremely detrimental to the stability of the GlnRS:tRNA<sup>Gln</sup> complex and thus efficient aminocaylation. This lowering of the efficiency of aminocaylation is reflected in vivo by the slower growth rate of <i>E. coli</i> cells harboring these GlnRS mutations as compared with those harboring specificity mutations (data not shown).

These results also raise the question of whether such differentiation of the identity set is applicable to other tRNA:AARS complexes where tRNA-dependent amino acid recognition has not yet been investigated. This may be particularly relevant since tRNA-dependent amino acid recognition was also observed for TrpRS which, in contrast to GlnRS, does not require the presence of tRNA for aminocayl-adenylate formation. For example, identity elements for both the yeast Asp (e.g. 43) and <i>E. coli</i> Ala (e.g. 44) systems have also been characterized at sub-saturating amino acid concentrations (45, 46) and thus might also be susceptible to comparable underestimations of <i>k<sub>cat</sub></i> to those described above for tRNA<sup>Gln</sup> variants.

<i>RNA</i>-Dependent Amino Acid Recognition Reduces the Error Rate of Aminocayl-tRNA Synthetases. The mechanism underlying RNA-dependent amino acid recognition by GlnRS is not yet clear as the existing structure was solved in the absence of glutamine. However, the recent crystallization of both wild-type and mutant tRNA<sup>Gln</sup>-GlnRS complexes in the presence of glutamine analogues should provide critical insights into the process of amino acid recognition (L. Silvian, V. Rath, and T. A. Steitz, personal communication). Nevertheless, the results presented here clearly show that specific interactions between the identity and recognition elements of the GlnRS:tRNA<sup>Gln</sup> complex determine the efficiency of glutamine recognition. The observation that aminocaylation of designed tRNAs by TrpRS results in an increase in the <i>K<sub>M</sub></i> of the cognate amino acid indicates the general applicability of tRNA-dependent amino acid recognition. This relationship effectively presents an additional kinetic barrier to the formation of mischarged tRNAs because aminocaylation by a cognate complex will be considerably more efficient than by a noncognate complex. In this context, tRNA-dependent amino acid recognition is comparable to the selection of cognate aminocayl-tRNAs during translation where two kinetic barriers also exist (47), but does not meet the criteria of proofreading as no pathway for the rejection of a noncognate is observed (48). Nevertheless, it serves a similar purpose to previously described proofreading pathways in that it effectively reduces the formation of incorrect products to a tolerable level.

The effectiveness of this mechanism as a means of increasing translational fidelity by reducing the error rate of GlnRS may be enhanced in vivo by competition, which also contributes to the accuracy of tRNA selection (49). Comparison of a number of studies concerning the concentration of amino acids both in Gram-negative bacteria in general (50) and <i>E. coli</i> in particular (51, 52) suggests that the cellular level of free glutamine is approximately 150 μM. This figure is supported by the general observation that physiological substrate concentrations are often below the <i>K<sub>M</sub></i> of the appropriate enzyme as this helps to optimize the rate of catalysis (53). Under such conditions, the cognate complex will be able to compete far more efficiently than the noncognate complex for the available glutamine, thus minimizing the generation of mischarged tRNAs. The necessity for a cognate complex able to compete effectively for available amino acid also provides a strong basis for the coevolution of tRNA identity and recognition elements. This requirement is reflected by the high degree of evolutionary conservation of the interactions that determine the accuracy of amino acid recognition in the GlnRS:tRNA<sup>Gln</sup> complex.

It is interesting to note that the same interactions optimize both glutamine recognition and the stability of the GlnRS: tRNA<sup>Gln</sup> complex, but whether this is a consequence or a determinant of the observed coevolution of the relevant RNA and protein sequences is unclear. Given the possibility that early aminocaylation events may have been accompanied by RNA alone (54) or by ribonucleoproteins where the RNA moiety was based on an acceptor-<i>Tyr</i>C ribonucleoprotein synthetase (16), in which case tRNA-dependent amino acid recognition would provide the main
impetus for the observed coevolution of the genes encoding tRNA^Glu and GlnRS.

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