Amino Acid-dependent Transfer RNA Affinity in a Class I Aminoacyl-tRNA Synthetase*

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Nathan T. Uter†, Ita Gruic-Sovulj§, and John J. Perona‡¶

From the †Department of Chemistry and Biochemistry, and ¶Interdepartmental Program in Biomolecular Science and Engineering, University of California, Santa Barbara, California 93106-9510.

Steady-state and transient kinetic analyses of glutaminyl-tRNA synthetase (GlnRS) reveal that the enzyme discriminates against noncognate glutamate at multiple steps during the overall aminoacylation reaction. A major portion of the selectivity arises in the amino acid activation portion of the reaction, whereas the discrimination in the overall two-step reaction arises from very weak binding of noncognate glutamate. Further transient kinetic experiments showed that tRNA\textsuperscript{Gln} binds to GlnRS ~60-fold weaker when noncognate glutamate is present and that glutamate reduces the association rate of tRNA with the enzyme by 100-fold. These findings demonstrate that amino acid and tRNA binding are interdependent and reveal an important additional source of specificity in the aminoacylation reaction. Crystal structures of the GlnRS:tRNA complex bound to either amino acid have previously shown that glutamine and glutamate bind in distinct positions in the active site, providing a structural basis for the amino acid-dependent modulation of tRNA affinity. Together with other crystallographic data showing that ligand binding is essential to assembly of the GlnRS active site, these findings suggest a model for specificity generation in which required induced-fit rearrangements are significantly modulated by the identities of the bound substrates.

The high fidelity of protein synthesis in living cells arises as a consequence of specificity at three distinct steps in the pathway: aminoacyl-tRNA formation, selection of aminoacyl-tRNA by elongation factor Tu, and ribosomal proofreading of the codon-anticodon interaction (1–3). Accuracy at the first aminoacyl-tRNA synthesis step is accomplished by the aminoacyl-tRNA synthetases, which attach amino acids to the corresponding tRNA species in a two-step reaction. Aminoacyl-tRNA synthesis step is accomplished by the aminoacyl-tRNA synthetase that achieves amino acid selectivity without use of an editing mechanism. *Escherichia coli* GlnRS synthesizes Gln\textsubscript{tRNA}\textsuperscript{Gln}, 10\textsuperscript{17}-fold more efficiently than Glu\textsubscript{tRNA}\textsuperscript{Gln}, corresponding to discrimination of 10.1 kcal/mol in favor of glutamine versus the nearly isosteric glutamate (6). Crystal structures of GlnRS have been solved in a ternary complex bound to tRNA and ATP, as well as in quaternary complexes bound to either cognate glutamine or noncognate glutamate in the presence of an ATP analog. The quaternary structures showed that the two amino acids bind in distinct orientations in the active site cleft (6, 7) and that a key arginine residue (Arg-30) may function as a negative determinant to drive glutamate binding into a nonproductive orientation via an apparently strong ionic interaction. This crystallographic finding predicts that a significant component of the strong amino acid selectivity might arise at the chemical rather than the binding step of the reaction.

Evidence exists suggesting that amino acid and tRNA binding to GlnRS are modulated by induced fit: mutually induced conformational changes in enzyme and substrates occur during initial binding and en route to a productive quaternary ground-state complex. GlnRS is one of four class I tRNA synthetases that require the presence of tRNA for catalysis of adenylate formation, suggesting that tRNA is required for proper active site assembly (8). Supporting this notion, comparisons of the

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§ Present address: Dept. of Biochemistry Faculty of Science, University of Zagreb, Strossmayerov trg. 14, 10000 Zagreb, Croatia.

¶ To whom correspondence should be addressed: Dept. of Chemistry & Biochemistry, University of California, Santa Barbara, CA 93106-9510.
 Tel.: 805-893-7369; Fax: 805-893-4120; E-mail: perona@chem.ucsb.edu.

1 The abbreviations used are: GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; DTT, dithiothreitol; QSI, 5'-O-[N-(t-glutaminyl)sulfamoyl] adenosine; Mes, 4-morpholinoethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; AMPCP, adenosine 5’-(α,β-methylene)triphosphate.
unliganded and tRNA-bound GlnRS structures shows that portions of both the amino acid and ATP binding sites are misoriented with respect to each other in the absence of tRNA (9, 10). Furthermore, fluorescence spectroscopy reveals ATP-dependent differences in binding of tRNA Glu and tRNA Gln to GlnRS that are suggestive of an induced-fit process (11, 12). Finally, kinetic data on modified GlnRS-tRNA complexes show that tRNA mutants in the distal anticodon and tertiary core regions can cause large decreases in both the \( k_{cat} \) for aminoacylation and the \( K_m \) for glutamine (13, 14). Pre-steady-state kinetics further demonstrated that aminoacylation of the tRNA Glu anticodon mutant U35A is decreased by 30-fold at the chemical step of the reaction and that glutamine binding affinity in the U35A mutant complex is weakened by 20-fold (15). These data demonstrate definitively that anticodon binding signals are transmitted some 40 Å to the GlnRS active site.

To explore the detailed origins of amino acid specificity in GlnRS, we performed steady-state and pre-steady-state kinetic studies utilizing both the conventional ATP-PP, exchange reaction, as well as a newer assay that relies upon internal 32P labeling of tRNA (16). This assay is highly sensitive and permits use of very high amino acid concentrations, a requirement for rigorous measurements of noncognate reactions in which ligand affinities are weak. We find that the specificity of GlnRS against glutamate in the two-step reaction is partitioned between the binding and catalytic steps of the reaction, with a greater component at the binding step. Furthermore, the pre-steady-state kinetic analysis uncovers a significant coupling between the amino acid and tRNA affinities of the enzyme, in which the identity of the bound amino acid (cognate or noncognate) controls how tightly tRNA Glu binds. This finding suggests that induced-fit structural pathways connecting the amino acid and tRNA binding sites may contribute to enzyme specificity. Thus, features of the enzyme architecture that control the required ligand-induced conformational changes may have a further role in helping to reject noncognate substrates. To date, GlnRS is the only tRNA synthetase for which noncognate aminoaoylation has been examined by both crystallographic and pre-steady-state kinetic analyses. Nonetheless, the common presence of induced-fit rearrangements among many tRNA synthetases, as revealed by x-ray structures in the apo- and liganded states, suggests that this mechanism may be quite general to the enzyme family.

**EXPERIMENTAL PROCEDURES**

**Enzyme Purifications**—T7 RNA polymerase was purified as described from E. coli BL21 cells containing the plasmid pAR1219 (6, 17). Purified enzyme was stored at \(-20 °C\) in a buffer containing 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM glycerol, 20 mM sodium phosphate (pH 7.8). A His6-C-terminal-tagged GlnRS was constructed by inserting the gls gene after the T7 promoter of the expression vector pSW1 (18). The His-tagged GlnRS was expressed in BL21-D3 (pLyS5) by induction with 1 mM isopropyl 1-thiogalactopyranoside at \( A_{600} \) between 0.4 and 0.6. Cells were resuspended in a buffer containing 0.5 mM NaCl, 50 mM HEPES (pH 7.2), 10 mM imidazole and were disrupted by French press. 100 mM phenylmethylsulfonyl fluoride, 100 mM benzamidine, 50 μl of RQI DNase (Promega), and 15 mM MgCl2 were added to the lysate, and the mixture was stirred at room temperature for 20 min. Lysed cells were then centrifuged for 30–45 min at 15,000 rpm, and the pellet was discarded. Enzyme was purified on a 5-mL nickel column (Amersham Biosciences) equilibrated in 10 mM imidazole, 20 mM HEPES (pH 7.2), 1 mM β-mercaptoethanol, and 0.5 mM NaCl; the elution buffer was identical to the equilibration buffer except for the addition of 120 mM imidazole. Eluted protein was dialyzed into storage buffer containing 50% glycerol, 20 mM potassium phosphate (7.4), 50 mM KCl, 2 mM DTT for 6–12 h. His-tagged GlnRS was recovered at better than 99% purity as judged by SDS-PAGE and was stored at high concentration at \(-20 °C\), as described for the native enzyme (6). GlnRS concentration was determined by absorbance \((E_{280}, 1 	ext{ mg/ml} = 1.06)\). Kinetic parameters of the His-tagged GlnRS were indistinguishable from that of native GlnRS within experimental error.

**tRNA Synthesis and Purification**—E. coli G-1 tRNA Glu (containing a catalytically neutral U1G mutation to promote efficient transcription initiation) was transcribed in high yield from a synthetic DNA template. The DNA template was constructed from complementary synthetic oligonucleotides containing a short central overlapping region, which were extended to form the full-length duplex by treatment with the Klenow fragment of E. coli DNA polymerase I (19). The DNA template incorporated 2′-O-methyl sugars at the two 5′-nucleotides in the non-coding DNA strand, resulting in a high proportion of enzymatically active tRNA transcripts. The reactions were then loaded onto a 5-mL DE-52 (Whatman) column, eluted, precipitated with ethanol, dried, and stored at \(-20 °C\). Prior to use the tRNA was resuspended and dialyzed against highly purified water.

**ATP-PP, Exchange Assay**—The reaction mixture for the ATP-PP, exchange assay consisted of 100 mM Mes-KOH (pH 6.0), 5 mM ATP, 20 mM MgCl2, 25 mM KCl, 4 mM DTT, 1 mM [32P]PP (10–50 μCi/ml). \( k_{cat} \) and \( K_m \) for glutamine and glutamate were determined by varying concentrations of these substrates from 0.05–50 mM and from 0.05–1.2 mM, respectively. The concentrations of GlnRS used were 5 μM for glutamate activation reactions, and 100 μM for glutamate activation reactions. In both cases, the concentration of tRNA Glu was 10 μM. The reactions were stopped by adding 1.5 μl of the reaction mixture to 3.0 μl of a solution containing 400 mM sodium acetate (pH 5.0), 0.1% SDS, 2.0 mg/ml lysozyme, and 0.1 mg/ml micrococcin P4000 (Amersham Biosciences). Kinetic parameters were obtained from at least three independent measurements. Initial velocities obtained by time-course analyses were plotted against substrate concentration and fit to the Michaelis-Menten equation with KaleidaGraph. \( k_{cat} \) and \( K_m \) were determined directly from these plots.

**Aminoacylation Assay and Steady-state Reactions**—Aminoacylation assays were carried out by a highly sensitive assay that has been recently described (6, 15, 16). Full-length tRNA Glu substrate prepared in a reaction mixture containing 0.1% SDS was labeled at the 3′-end of the nucleotide linkage using α-32P-labeled ATP and the exchange reaction of tRNA nucleotidyltransferase (6). To prepare the tRNA sample for use in kinetic assays, labeled and unlabeled substrate were mixed to the appropriate final concentration, heated at 65 °C for 3 min, and then cooled at room temperature in the presence of 7.5 mM MgCl2. All reactions were quenched in a buffer containing 0.1% SDS, 0.15 mM ATP, and 50 mM Tris (pH 7.2). P1 nuclease digestions were performed by adding 1–5 μl of the reaction mixture to a microwell containing 3–5 μl of 0.1 mg/ml P1 nuclelease (Fluka), 0.15 mM sodium phosphate (pH 5.2) and incubating 10 min at ambient temperature. Aminoacylated tRNA (as 3′-aminoacylated A76) and nonreacted substrate (as unmodified AMP) were separated by TLC and quantitated by phosphorimaging analysis, as described before (6).

Plateau aminoacylation levels were determined directly by the ratio of aminoacyl-A76 and unmodified A76 after P1 nuclease treatment and TLC separation (6). All steady-state kinetic reactions were performed at 37 °C in a buffer containing 10 mM ATP, 5 mM DTT, 50 mM Tris (pH 7.3), and 15 mM MgCl2. Concentration ranges of glutamine and tRNA Glu used to determine Michaelis parameters for cognate glutaminylation reactions were as reported (15). To determine \( k_{cat} \) and \( K_m \) for tRNA Glu for glutaminylation reactions, the reactions were performed at 0.3 μM GlnRS and 2 μM glutamate. The tRNA Glu concentration was varied between 1.4 and 56 μM. For both cognate and noncognate steady-state reactions, time courses for product formation were replotted using Eadie-Hofstee and hyperbolic (Equation 4) analysis to derive \( k_{cat} \) and \( K_m \).

**Rapid Chemical-quench Kinetics**—Single-turnover reactions were performed under conditions of 5-fold enzyme excess under solution and temperature conditions identical to the steady-state reactions. Measurements of cognate glutaminyltRNA synthetase reactions were performed using a rapid chemical-quench flow apparatus (Kintek RQF-3) as described elsewhere (15). Noncognate misaminoacylation reactions were slow enough to allow sampling by hand. All reactions were quenched in 50 μl of 20 mM sodium acetate (pH 7.2) and 0.1% SDS. 8–10 time points were collected for each \( k_{cat} \) determination. The amino acid \( K_m \) values under single-turnover conditions were determined by titrating the concentration of the amino acid while maintaining saturating levels of ATP and tRNA. The glutamine \( K_m \) value in cognate reactions was previously determined as described (15). For noncognate glutamate, the \( K_m \) for glutamate was evaluated at saturating concentrations of the other substrates: 10 mM ATP, 30 μM tRNA Glu, and 150 μM GlnRS. Titrations
to assess $K_m$ for tRNA$^{Gln}$ were performed at 10 mM ATP and either 10 mM glutamine or 2 mM glutamate. All data were acquired in triplicate. Burst kinetics analyses in pre-steady-state experiments for noncognate glutaminyl-tRNA synthetase were performed similarly to those for cognate reactions (15), at concentrations of 0.3 μM GlnRS, 27 μM tRNA$^{Gln}$, 3 μM glutamate, and 10 mM ATP.

**Pulse-chase Experiments**—Pulse-chase trap experiments for cognate glutaminyl-tRNA synthetase were performed as described above for the single-turnover rapid quench experiments, with the following exceptions. The concentration of tRNA$^{Gln}$ was kept constant at 13 nM, whereas the concentration of GlnRS was varied between 40 and 120 nM. Bulk concentration of tRNA$^{Gln}$ was kept constant at 13 nM, whereas the solution of P1 nuclease prepared as described above. Noncognate reactions (Equation 2), where $O$ is time, $t$ is time, and AA is the fraction of tRNA$^{Gln}$ aminoacylated, or % aminoacylation as described above.

The rate constants $k_{cat}$, $k_{on}$, and $k_{off}$ are to be interpreted according to the reaction depicted in the simplified reaction for data analysis shown in Fig. 3, where $k_{on}$ and $k_{off}$ refer to the association and dissociation rates, respectively, for either ATP, amino acid, or tRNA$^{Gln}$. $k_{chom}$ is a composite intrinsic rate constant for the two-step aminoacylation reaction.

The amplitude of aminoacylation was determined by the fit for each time course. Burst kinetics values were fit by Equation 3.

The kinetic $K_m$ for tRNA$^{Gln}$ in the presence of glutamate was calculated by reploting GlnRS concentration against the $k_{cat}$ for that concentration. These data were then fit to either a hyperbolic (Equation 4) or quadratic binding equation (Equation 5) according to the concentration of tRNA$^{Gln}$ used in the reactions:

$$Y = (E_0 - k_{chom}/E_0 + K_a)$$

(Eq. 4)

$$k_{chom} = (O^t((E + T + K_d) - ((E + T + K_d) - (4*E^2*Y^3)/(2*Y^2))^{1/2})$$

(Eq. 5)

Here $E$ is the concentration of GlnRS, $T$ is the concentration of tRNA$^{Gln}$, and $O$ is the amplitude.

Time courses for pulse-chase experiments, performed under conditions where enzyme is in molar excess to tRNA, were fit to a single-exponential function. The enzyme concentration was then replotted against the observed rate ($k_{obs}$) for catalysis (Equation 6). Under circumstances where $k_{cat}$ is very small, the slope $k_{obs}/[E]$ reduces to the rate of association ($k_{on}$). However, if the magnitudes of $k_{off}$ and $k_{chom}$ are similar, then the value of $k_{obs}/[E]$ sets a lower limit on the rate of association.

$$k_{obs}/[E] = (k_{cat})(k_{chom})(k_{chom} + k_{cat})$$

(Eq. 6)

Kinetic data exhibiting a prominent lag phase was fit using the reaction scheme described above, using the program Scientist. In these reactions ATP and glutamine saturate the enzyme complex, and the tRNA$^{Gln}$ binding equilibria account for the rate-limiting interactions that are indicated by appearance and disappearance of the lag. Global fits using nonlinear regression analysis were used to define values for the amplitude and for each of the distinct rate constants $k_{cat}$, $k_{off}$, and $k_{chom}$ reported in Table II.

**RESULTS**

**Discontinuation against Glutamate at the Amino Acid Activation Step**—To evaluate the contribution of the aminoacyl adenylate synthesis step to the overall accuracy of glutamine, the rate of exchange of 23P-labeled pyrophosphate into ATP was measured in the presence of either cognate glutamine or noncognate glutamate (Table I). Unlike most tRNA synthetases, GlnRS does not synthesize the aminoacyl adenylate intermediate in the absence of tRNA. We therefore first searched for reaction conditions in which transfer of amino acid to the tRNA would be sufficiently slow to permit measurement of ATP-PP$_1$ exchange. This revealed that the proportion of radiolabelled ATP synthesized increases substantially at pH 6.0, where amination efficiency is reduced (Fig. 1). This is the case even though the velocity of ATP-PP$_1$ exchange also decreases at the lower pH values, as may be seen from the fact that reactions at lower pH take a longer time to reach plateau levels. Reactions at pH 6.0 were conducted in the presence of either glutamine or noncognate glutamate, and the steady-state velocity of ATP formation was monitored (Fig. 1 and Table I). Interestingly, the $K_m$ of 3.2 mM for glutamine in this reaction is 16-fold higher than the $K_m$ of 0.2 mM measured for the two-step glutaminyl-tRNA synthetase reaction (15). The elevation in $K_m$ may indicate a change in the glutamine binding affinity at lower pH; alternatively, because the reaction mechanisms differ in the two assays, the distinction may reflect instead an alteration in other kinetic parameters (22). In the presence of tRNA$^{Gln}$, rapid exchange between ATP and PP$_1$ occurred with $k_{cat}$ of 47 s$^{-1}$ (Table I), significantly faster than the steady-state rate of Gln$^+$tRNA$^{Gln}$ formation in the two-step reaction ($k_{cat} = 3.2$ s$^{-1}$ (14)). This is expected given that dissociation of Gln$^+$tRNA$^{Gln}$ is known to be the rate-limiting step in the aminoacylation pathway (15).

The ATP-PP$_1$ exchange reaction was also performed in the presence of glutamate. Significantly impaired synthesis of glutamyl adenylate was observed in comparison to the cognate reaction (Table I). The $K_m$ for glutamate is increased by 10$^2$-fold compared with glutamine, whereas $k_{cat}$ is decreased by at most 10$^3$-fold. It is known that the level of discrimination against glutamate in the overall amination reaction is 10$^2$-fold, as assessed by the relative values of $k_{cat}/K_m$. Thus, a major component (>4 × 10$^4$-fold) of this selectivity arises in the first activation step of the reaction.

**Partitioning of Amino Acid Discrimination between Binding and Catalytic Steps**—A novel, highly sensitive assay for tRNA aminoacylation was applied to evaluate the kinetic origins of amino acid discrimination by E. coli GlnRS. The assay is based on 23P-labeling at the 3′-internucleotide linkage of the tRNA (16). After aminoacylation, the labeled tRNA is degraded by P1 nuclease, resulting in a mixture of labeled aminoacyl-AMP (product) and labeled AMP (unreacted substrate). These two compounds are then separated by TLC and quantified by image
analysis (Fig. 2). Previously, we used the new assay to determine $k_{\text{cat}}/K_m$ for cognate glutamination and for formation of misacylated Glu-tRNA\textsuperscript{Gln} by GlnRS (6). These data showed that GlnRS discriminates against noncognate glutamate by 10\(^5\)-fold, or 10.1 kcal/mol in the transition state for aminocoylation. Steady-state parameters for glutaminylation were identical to those previously determined with the conventional assay ($k_{\text{cat}} = 3.2$ s\(^{-1}\); $K_m$ (tRNA) = 0.31 $\mu$m), verifying that the new experimental approach is suitable for quantitative kinetic studies (13, 15).

The crystal structure of the GlnRS-tRNA\textsuperscript{Gln} complex bound to glutamate and an ATP analog showed that the noncognate amino acid binds in a distinct orientation, in which the $\alpha$-carboxylate is displaced by $-1$ Å as compared with the position occupied by the equivalent moiety of cognate glutamine (6). This finding suggested that a significant component of amino acid discrimination resides at the chemical step of the reaction rather than in the initial binding. To evaluate this hypothesis, we examined noncognate aminocoylation by single-turnover kinetics, in which reactions were carried out with molar excess of enzyme over tRNA (Fig. 2). These noncognate reactions were sufficiently slow to allow sampling by hand, in contrast to cognate glutaminylation, which required rapid mixing (15). In GlnRS, formation of the activated aminocoyl adenylate intermediate requires the presence of tRNA (1), making isolation of the second tRNA transfer step difficult. Therefore, for these reactions we measured a composite $k_{\text{chem}}$ (Fig. 3), which represents the two-step reaction using ATP, amino acid, and tRNA\textsuperscript{Gln} substrates and generating aminocoyl-tRNA\textsuperscript{Gln}, AMP, and PP.

The concentrations of ATP, glutamate, and tRNA\textsuperscript{Gln} were systematically varied to determine conditions for substrate saturation. For cognate reactions performed in the rapid quench apparatus, systematic mixing controls previously established that the rate of product formation does not depend upon which substrates are preincubated with enzyme (15). Saturation for glutaminylation was established at 3 $\mu$m tRNA\textsuperscript{Gln}, 10 mM glutamine, and 10 mM ATP; under these conditions a single-exponential fit of the data yields a maximal $k_{\text{chem}}$ of 28 s\(^{-1}\) (15) [Table II]. This represents the rate of all reaction steps up to and including product formation on the enzyme. For the noncognate reaction saturation was estabilished at 10 mM ATP and at 33 $\mu$m tRNA\textsuperscript{Gln}. However, titration of the glutamate concentration across a wide range showed that for this substrate the rate of product formation increases linearly up to 2 M without exhibiting saturation (Fig. 4A). Control experiments demonstrated that the $k_{\text{cat}}$ of cognate reactions is reduced by only 2-fold in the presence of 2.7 M NaCl; this effect is small, but may nonetheless limit detection of binding saturation at very high glutamate concentrations. Because saturation is not established, $k_{\text{chem}}$ for glutamoylation determined at the highest glutamate concentration tested represents a lower limit for the reaction chemistry ($k_{\text{chem}} > 0.17$ s\(^{-1}\); Table II). Thus, GlnRS discriminates against glutamate at the chemical steps by at most 165-fold. The inability to detect glutamate saturation precludes determination of the glutamate $K_m$ parameter in the steady-state reaction (Table II; the $k_{\text{cat}}$ is determined with reference to tRNA\textsuperscript{Gln}, see below).

The inability to saturate glutamate in the two-step aminocoylation is in contrast to the saturation that is observed in the ATP-PP\(_i\) exchange assay, in which a $K_m$ value of 252 mM could be determined (Fig. 4B and Table I). Possibly, this distinction is due to the significantly lower pH at which the ATP-PP\(_i\) reaction has been performed (pH 6.0 versus pH 7.5 in the two-step aminocoylation). Although only a small fraction of glutamate is expected to be protonated at pH 6.0, protonation may be favored in the environment of the active site. Protonated glutamate would better resemble glutamine and would be expected to bind better than the negatively charged species (see “Discussion”).

The dependence of the single-turnover reaction rate on the concentration of amino acid, in the presence of saturating levels of the other substrates, allows determination of the amino acid binding affinity by a hyperbolic fit of $k_{\text{chem}}$ with concentration. For cognate glutamine, amino acid binding is in rapid equilibrium with the enzyme (15); under these conditions, the $K_d$ of 1.1 mM determined by this kinetic approach is equivalent to the equilibrium binding constant (22). Because no saturation is observed for glutamoylation, only a lower limit for the amino acid binding affinity can be established. From the observation that the reaction rate increases linearly up to 2 M glutamate (Fig. 4), we roughly estimate $K_d$ (glutamate) to be above 750 mM. Thus, amino acid discrimination by GlnRS at the binding step may approach or exceed 10\(^6\)-fold, so that specificity is apportioned somewhat more at the binding rather than the catalytic step. The concentration of glutamate in vivo is at the millimolar level, so the very weak affinity will certainly play the major role in precluding significant levels of Glut-tRNA\textsuperscript{Gln} synthesis in the cell.

For cognate reactions, we previously showed that the rate-limiting step in the steady state is release of the Glut-tRNA\textsuperscript{Gln} product. This is established by the observation of a rapid burst of Glut-tRNA\textsuperscript{Gln} synthesis followed by a slow steady-state rate (15) [Fig. 5]. In the case of the noncognate reactions, however, no burst could be observed under conditions where the first turnover can be readily detected (Fig. 5b), although the possibility of a shallow burst at very early time points cannot be excluded. However, the much slower rate observed in single-turnover reactions for synthesis of Glut-tRNA\textsuperscript{Gln} would be consistent with an early rate-limiting step in the reaction pathway (Table II).

tRNA\textsuperscript{Gln} Binding Affinity Determined by Transient Kinetics—The dependence of the single-turnover reaction rate on tRNA concentration was measured as a means of determining
the complex, the association rate possible for glutamine binding (15)). At high concentrations of aminoacylation in which the amino acid is transferred to tRNA. For the two-step cognate reaction by wild-type GlnRS, $k_{\text{on}}$ and $k_{\text{off}}$ indicate the association and dissociation rate constants, respectively, for any of the substrates. The over-all scheme (top) describes the full two-step aminoacylation reaction. To simplify the drawing, in this scheme $k_{\text{on}}$ and $k_{\text{off}}$ indicate the association and dissociation rate constants, respectively, for any of the substrates. $k_{\text{act}}$ is the rate constant for synthesis of the aminoacyl adenylate; $k_{\text{cat}}$ for ATP-PP$^i$ exchange (Table I; Fig. 1) measures the reverse of this reaction. $k_{\text{reacta}}$ is the forward rate constant for the second step of aminoacylation in which the amino acid is transferred to tRNA. For the two-step cognate reaction by wild-type GlnRS, $k_{\text{cat}}$ corresponds to product release, as shown. In the simplified scheme (bottom), $k_{\text{chem}}$ is a composite rate constant for the two-step reaction (Fig. 2); $k_{\text{on}}$ and $k_{\text{off}}$ are determined from substrate titrations under single-turnover conditions (see text and “Experimental Procedures”).

Fig. 2. Time course for single-turnover glutamyl-tRNA synthesis by E. coli GlnRS. Conditions were: 50 mM Tris (pH 7.5), 15 mM MgCl$_2$, 5 mM DTT, 10 mM ATP, 2 mM glutamate, 33 $\mu$M tRNA$^{\text{Gln}}$, and 71 $\mu$M GlnRS. The inset shows the imaged TLC plate at the respective time points. The fraction aminoacylated is determined directly by the ratio of intensities for Gln-AMP and AMP.

**Reaction Scheme:**

\[
\begin{align*}
&\text{QES+ATP+\text{tRNA}} \\
&\quad \xrightarrow{k_{\text{act}}} \text{QES+ATP+\text{tRNA}} \xrightarrow{k_{\text{chem}}} \text{QES+\text{AMP+}\text{tRNA}} \\
&\text{QES+\text{AMP+}\text{tRNA}} \xrightarrow{k_{\text{off}}} \text{QES+ATP+\text{tRNA}} \xrightarrow{k_{\text{on}}} \text{QES+\text{AMP+}\text{tRNA}}
\end{align*}
\]

**Simplified reaction scheme used for analysis of two-step single-turnover reactions:**

\[
\begin{align*}
&\text{QES+ATP+\text{tRNA}} \\
&\quad \xrightarrow{k_{\text{on}}} \text{QES+ATP+\text{tRNA}} \xrightarrow{k_{\text{chem}}} \text{QES+\text{AMP+}\text{tRNA}} \xrightarrow{k_{\text{off}}} \text{QES+ATP+\text{tRNA}}
\end{align*}
\]

As an independent approach to establish whether these values of the microscopic rate constants adequately describe the reaction mechanism, we performed pulse-chase experiments. In some systems, pulse-chase experiments allow the direct calculation of $k_{\text{on}}$ and $k_{\text{off}}$ rates for the titrated substrate. Pulse-chase experiments reveal important information about the actual mechanistic details in catalysis in both a qualitative and quantitative fashion (see below). The methodology for pulse-chase is analogous to that of the single-turnover experiments described above, except that the quenching step is performed by addition of a large molar excess of unlabeled bulk tRNA enriched in tRNA$^{\text{Glu}}$ (21). This quenching procedure does not denature the enzyme, so that bound, unreacted tRNA partitions between dissociation and aminoacylation (25, 26). By contrast, the denaturing quench blocks all further forward progress of the reaction.

In these experiments, the concentration of labeled tRNA$^{\text{Gln}}$ transcript was kept constant at 13 nM, whereas the concentration of GlnRS was varied from 40 to 240 nM (Fig. 8). For each reaction, we found that product formation fits well to a single-exponential function. A replot of $k_{\text{obs}}$ versus enzyme concentration...
Values derived by allowing all variables to float in the global fitting, and values derived by fixing $k_{\text{on}}$ and $k_{\text{chem}}$. Errors for these parameters were taken as the standard deviations of these estimates from the mean.

The $K_d$ reported is estimated from global fitting. $K_d$ determined by directly replotting the data on a hyperbolic binding isotherm is 6.7 µM (see Fig. 8).

Amino Acid Specificity in Glutaminyl-tRNA Synthetase

### Table II

| Glutamate | Glutamine |
|-----------|-----------|
| $a$ | $b$ |

| Parameter | Value (nm) | Value (µM) |
|-----------|------------|------------|
| $K_{\text{cat}}$ (tRNA<sup>Glutam</sup>) | 0.036 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |
| $K_{\text{cat}}$ (tRNA<sup>Glutam</sup>) | 0.36 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |
| $K_{\text{on}}$ (tRNA<sup>Glutam</sup>) | 0.43 ± 0.14<sup>c</sup> | 50 ± 10<sup>a</sup> |
| $K_{\text{off}}$ (tRNA<sup>Glutam</sup>) | 0.36 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |
| $K_{\text{on}}$ (a) | 1.1 ± 0.00<sup>d</sup> | 4.7 ± 1.3 × 10<sup>e</sup> |
| $K_{\text{off}}$ (a) | 0.36 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |
| $K_{\text{on}}$ (b) | 43 ± 14<sup>c</sup> | 4.7 ± 1.3 × 10<sup>e</sup> |
| $K_{\text{off}}$ (b) | 0.36 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |
| $K_{\text{on}}$ (c) | 1.1 ± 0.00<sup>d</sup> | 4.7 ± 1.3 × 10<sup>e</sup> |
| $K_{\text{off}}$ (c) | 0.36 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |
| $K_{\text{on}}$ (d) | 43 ± 14<sup>c</sup> | 4.7 ± 1.3 × 10<sup>e</sup> |
| $K_{\text{off}}$ (d) | 0.36 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |
| $K_{\text{on}}$ (e) | 1.1 ± 0.00<sup>d</sup> | 4.7 ± 1.3 × 10<sup>e</sup> |
| $K_{\text{off}}$ (e) | 0.36 ± 0.10<sup>b</sup> | 12.2 ± 2.7<sup>e</sup> |

a Previously reported in Ref. 6.  
<sup>a</sup>Previously reported in Ref. 15.  
<sup>c</sup>The $K_{\text{off}}$ for glutamate in glutamylation could not be determined because saturation was not observed.  
<sup>b</sup>Values reported for $k_{\text{on}}$(tRNA<sup>(+)</sup>) and $k_{\text{off}}$(tRNA<sup>(−)</sup>) are the average of values derived by allowing all variables to float in the global fitting, and values derived by fixing $k_{\text{on}}$ and $k_{\text{chem}}$. Errors for these parameters were taken as the standard deviations of these estimates from the mean.

The rate of association for tRNA<sup>Glutam</sup> is at or very near to that expected if diffusion is controlling, indicating very rapid binding of the tRNA under conditions of the enzymatic reaction.  

![Fig. 4](image)

**Fig. 4.** a, dependence of reaction rates on amino acid concentration. Replots of amino acid concentration versus $k_{\text{chem}}$ for glutaminylamination (panel a at left (15)) and glutamylation (panel b at right). $k_{\text{chem}}$ is equivalent to the composite rate constant $k_{\text{chem}}$ (see reaction scheme in “Experimental Procedures”). Glutamine is in rapid equilibrium with the GlnRS-tRNA<sup>Glutam</sup> complex, allowing determination of $K_{\text{cat}}$ from a hyperbolic fit (Equation 4) of the curve (15) (Table II). The replot of glutamate concentration versus $k_{\text{chem}}$ shows that the enzyme complex is not saturated at concentrations up to 2.0 M. b, saturation of glutamate binding at pH 6.0 in ATP-PP<sub>1</sub> exchange. Hyperbolic replot of $k_{\text{chem}}$ versus glutamate concentration ($r$ = 0.985) demonstrating saturation at pH 6.0. 

Because of partitioning between tRNA dissociation and the forward reaction (defined by the $(k_{\text{chem}})/(k_{\text{chem}} + k_{\text{off}})$ term in Equation 6; see “Experimental Procedures”), this slope sets a lower limit for the association rate $k_{\text{on}}$(tRNA<sup>(+)</sup>). Using the estimates of $k_{\text{on}}$(tRNA<sup>(+)</sup>) and $k_{\text{chem}}$ derived from global fitting, the parameter $(k_{\text{chem}})/(k_{\text{chem}} + k_{\text{off}})$ is estimated at 0.44, yielding in turn an estimate of $k_{\text{off}}$(tRNA<sup>(−)</sup>) = $3.8 \times 10^9$ M<sup>−1</sup>s<sup>−1</sup>. This is within 3-fold of the rate determined by global fitting of the denaturing-quinch reactions. In principle, $k_{\text{on}}$(tRNA<sup>(+)</sup>) and $k_{\text{off}}$(tRNA<sup>(−)</sup>) can be determined from the pulse-chase data using the relation $[P]_{\text{obs}}/[P]_0 = (k_{\text{chem}})/(k_{\text{chem}} + k_{\text{off}})$, where $[P]_{\text{obs}}$ is the amount of product formed at the reaction time point where the concentration of the GlnRS<sup>−</sup>tRNA<sup>Glutam</sup> complex is maximal (25). However, simulations showed that maximal accumulation of the complex occurs very rapidly, within the dead-time of the rapid quench instrument. Thus, an accurate estimate of $[P]_{\text{obs}}$ could not be made.

Amino Acid-dependent tRNA Binding Affinity—We next examined the dependence of tRNA binding on the identity of the bound amino acid. Steady-state kinetic measurements first showed that tRNA<sup>Glutam</sup> binding does approach saturation for noncognate glutamylation reactions carried out at 2 M glutamate. The $K_m$ for tRNA<sup>Glutam</sup> is determined to be 19 µM, some 60-fold higher than its $K_m$ in the cognate reactions. Because the tRNA saturates, we could also determine an apparent $k_{\text{cat}}$, which is decreased by only 70-fold compared with the cognate reaction. In contrast with the cognate reactions, single-turnover glutamylation time courses using the denaturing quench methodology failed to reveal a detectable lag phase at any tRNA concentration (Fig. 9). It thus appears that for noncognate glutamylation the formation of the GlnRS<sup>−</sup>tRNA<sup>Glutam</sup> complex is in rapid equilibrium ($k_{\text{off}}$(tRNA<sup>(−)</sup>) $\gg$ $k_{\text{chem}}$), allowing determina-
tion of $K_d$ by fitting the plot of $k_{obs}$ versus enzyme concentration to a standard hyperbolic isotherm. This yields $K_d$ of 6.7 $\mu$M for tRNA$^{\text{Gln}}$ (Fig. 10).

The data were also subjected to global fitting to estimate the values of the association ($k_{on(tRNA)}$) and dissociation rate constants ($k_{off(tRNA)}$) for tRNA$^{\text{Gln}}$ (Fig. 11). Nonlinear least-squares regression analyses were again performed by floating all three of the variables $k_{on}$, $k_{off}$, and $k_{chem}$, yielding dissociation rate ($k_{off(tRNA)}$) of 60 $s^{-1}$, similar to the dissociation rate for the cognate reaction (Table II). However, the derived association rate of $4.5 \times 10^6$ $M^{-1} s^{-1}$ is ~100-fold lower than for the cognate reaction, suggesting that structural rearrangements upon tRNA binding may be perturbed in the formation of the

FIG. 5. Pre-steady-state kinetics. a, time course of glutamylation under pre-steady-state conditions (see text), b, time course of glutamylation under pre-steady-state conditions, showing a clear rapid burst of Gln-tRNA$^{\text{Gln}}$ product in the first enzyme turnover followed by a slower steady-state rate (Equation 3) (15).

FIG. 6. tRNA concentration dependences in glutaminylation. Time courses showing concentration dependences for the formation of a lag phase in single-turnover reactions (see text). This is characteristic of a two-step reaction pathway where the binding phase becomes observable at lower concentrations of complex, and ultimately becomes rate-limiting at the lowest concentrations where a lag is no longer evident. GlnRS/ tRNA$^{\text{Gln}}$ concentrations are as follows: 2.5/0.5 $\mu$M (●), 0.5/0.1 $\mu$M (□), 0.24/0.013 $\mu$M (+), 0.08/0.013 $\mu$M (×), 0.04/0.013 $\mu$M (+), and 0.005/0.0001 $\mu$M (▲). Reactions corresponding to GlnRS concentrations of 0.24 mM, 0.08 mM, and 0.04 mM show discernable lags and are fit to double exponentials (Equation 2), although the other time courses fit well to a single-exponential function (Equation 1). Data for each reaction were taken in additional 0.5-s time intervals to a final time of 3 s; the longer time points are omitted to allow clearer display of the lags at short times.

FIG. 7. Global fitting of a set of single-turnover GlnRS reactions. Reactions were carried out at constant tRNA concentration and varying amounts of enzyme in molar excess. tRNA$^{\text{Gln}}$ is the limiting substrate (13 nM), whereas ATP and glutamine are present at saturating levels. The concentration of GlnRS was varied as follows: 240 nM (■) ($R = 0.997$), 160 nM (▲) ($R = 0.999$), 80 nM (●) ($R = 0.998$), and 40 nM (○) ($R = 0.998$), and data were subjected to nonlinear squares regression analysis (see text). Data for each reaction were taken in additional 0.5-s time intervals to a final time of 3 s; the longer time points are omitted to allow clearer display of the lags at short times.

FIG. 8. Pulse-chase glutaminylation reactions. Single-turnover measurements of glutaminylation quenched by the addition of bulk tRNA (pulse-chase). GlnRS was varied from 240 nM (×), 160 nM (○), 80 nM (■), and 40 nM (●), whereas tRNA$^{\text{Gln}}$ was kept constant at 13 nM. Each time course was fit to a single-exponential function to derive the pseudo-first-order rate constant (Equation 1). The inset shows concentration dependence of $k_{chem(\text{obs})}$ versus GlnRS concentration, where the slope sets the lower limit to $k_{on}$ ($1.7 \times 10^8$ $M^{-1} s^{-1}$) ($R = 0.991$).

FIG. 9. Misaminoacylation of tRNA$^{\text{Gln}}$ with glutamate by GlnRS. The plots show GlnRS/tRNA$^{\text{Gln}}$ concentrations of 0.6 $\mu$M/0.12 $\mu$M (●), 0.3/0.06 $\mu$M (□), 0.15/0.03 $\mu$M (○), and 0.075/0.015 $\mu$M (×) fit to a single-exponential (Equation 1). Insets shows reactions, 0.15/0.03 $\mu$M (△) and 0.075/0.015 $\mu$M (×), which were closely monitored at early time points. No lag could be detected for any of the reactions.

The data were also subjected to global fitting to estimate the values of the association ($k_{on(tRNA)}$) and dissociation rate constants ($k_{off(tRNA)}$) for tRNA$^{\text{Gln}}$ (Fig. 11). Nonlinear least-squares regression analyses were again performed by floating all three of the variables $k_{on}$, $k_{off}$, and $k_{chem}$, yielding dissociation rate ($k_{off(tRNA)}$) of 60 $s^{-1}$, similar to the dissociation rate for the cognate reaction (Table II). However, the derived association rate of $4.5 \times 10^6$ $M^{-1} s^{-1}$ is ~100-fold lower than for the cognate reaction, suggesting that structural rearrangements upon tRNA binding may be perturbed in the formation of the
DISCUSSION

Pre-steady-state Kinetics by Chemical Quench—In this work we describe the application of both the conventional ATP-PP₆ exchange assay, as well as a more novel chemical-quench methodology to examine the origins of amino acid discrimination in a tRNA synthetase by pre-steady-state kinetics (6, 15, 16). The most important new feature of the chemical-quench assay is the use of ³²P-labeled tRNA. This gives excellent sensitivity while also permitting the use of very high concentrations of unlabeled amino acid. This approach should be readily generalizable to all tRNA synthetases, and will likely require only minor modifications to optimize TLC separations for the different aminoacyl adenylates. In addition, this assay offers a convenient method for measuring aminoacylation kinetics toward nonstandard amino acid substrates, most of which are not commercially available in radiolabeled form.

We demonstrate now that the chemical-quench assay is suitable for the reliable determination of both steady-state and microscopic kinetic parameters for aminoacylation, including estimation of ligand binding affinities and tRNA association and dissociation rates. This greatly benefits structure-function analyses, because for many applications it may reduce or eliminate the need to implement distinct binding assays. Moreover, it is important to note that the estimates of ligand affinities by these kinetic techniques yield values that are directly relevant to the enzyme under catalytic conditions. All other approaches to determining ligand affinity require that the reaction be blocked, either by eliminating a required substrate or cofactor, or by performing the reaction under noncatalytic conditions.

As a further control for the effects of the high ionic strength in these reactions performed at very high glutamate concentrations, we performed single-turnover reactions by the denaturing quench protocol at 4-fold lower glutamate concentrations. We find that the kinetic $K_d$ under these conditions is 9.8 µM (data not shown), very similar to the value derived from the measurements at 2 mM glutamate (Table II). Clearly, the significant effect on tRNA<sub>Gln</sub> affinity caused by the presence of glutamate is not a consequence of weakened interactions due to high ionic strength. Instead, weakened tRNA affinity in the presence of the noncognate amino acid represents an inherent additional mechanism to ensure cognate aminoacyl-tRNA synthesis by GlnRS. We are unaware of previous studies demonstrating that tRNA affinity by a tRNA synthetase depends upon the identity of the bound amino acid.

Clearly, the presence of noncognate glutamate substantially alters the interaction of GlnRS with tRNA<sub>Gln</sub>. The slow association rate in these reactions suggests that the tRNA-enzyme complex may undergo a slow conformational transition after initial docking, in the presence of the noncognate amino acid. Although the crystal structure of the GlnRS<sub>tRNA<sub>Gln</sub></sub> complex bound to glutamate does not reveal misorientation of the tRNA, it is possible that any rearrangement in the final bound state is small, and may be masked by the lattice forces that drive an orientation for the tRNA that is both productive for catalysis and also favorable for crystal formation (6, 28).

The different behavior observed in the noncognate reactions may be explained with reference to the replot of observed rates in the pulse-chase reactions, versus enzyme concentration (Fig. 12, inset). This replot yields a slope, $k_{diss}/E = (k_{diss})/(K_0 + k_{diss})$, of 1.5 $\times$ 10⁳ M⁻¹ s⁻¹, which sets a lower limit for the tRNA association rate. In contrast to the cognate reaction, where bound tRNA has roughly equal probability of partitioning back to dissociation or forward to reaction, in this case the rate constant for dissociation estimated from global fitting is much greater than the rate constant for the forward aminoacylation reaction (Table II). The ratio $(k_{diss})(k_{diss} + k_{diss})$ estimated from the global fitting parameters is ~0.005; thus, the observed slope $k_{diss}/E$ in the pulse-chase reactions is some 100-fold lower than the association rate constant. The slow association in noncognate reactions as well as the favored partitioning toward dissociation result in very little buildup of the pre-reactive complex; hence, little distinction is observed between reactions performed by the denaturing quench versus the pulse-chase methodologies.

FIG. 10. Amino acid dependent tRNA<sub>Gln</sub> affinity. Replot of GlnRS concentration versus $k_{chem}$ for glutamylation to derive the $K_f$ for tRNA ($R = 0.981$).

FIG. 11. Global fitting of a set of single-turnover glutamylation reactions. Global fitting of the single-turnover GlnRS reactions for noncognate glutamylation, where tRNA<sub>Gln</sub> is the limiting substrate (0.135 µM) and ATP and glutamine are at saturating levels. The concentration of GlnRS was varied as follows: 32.5 µM (R = 0.999), 26 µM (R = 0.998), 130 µM (R = 0.997), and 6.5 µM (R = 0.999), 3.25 µM (R = 0.991), and 1.3 µM (R = 0.985). Data were subjected to nonlinear least squares regression analysis (see text). Data for each reaction were taken in additional 10-s time intervals (not shown) to a final time of 30 s.

noncognate quaternary complex. As observed for the cognate reactions, the derived value of $k_{chem}$ is again similar to that measured experimentally, although some increased uncertainty exists here due to the lack of glutamate saturation (Table II; $k_{chem} > 0.17$ s⁻¹). The ratio of the association and dissociation rate constants yields $K_f$ for tRNA<sub>Gln</sub> of 12.3 µM. This represents 60-fold weaker affinity for tRNA than in the cognate reaction, and is within 2-fold of the value determined in the hyperbolic replot.

To further examine noncognate glutamylation, we again performed single-turnover reactions using a cold-quench solution containing excess tRNA (Fig. 12). Application of this methodology to the cognate reactions had revealed that the reaction rates under equivalent conditions were very much faster for the noncognate quaternary complex that bound tRNA has roughly equal probability of partitioning to nonlinear least squares regression analysis (see text). Data for each reaction were taken in additional 10-s time intervals (not shown) to a final time of 30 s.

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such as lowered pH. In favorable circumstances, such as when a reliable fluorescence-based binding assay is available, comparisons of ligand affinities determined by kinetics and by an alternative technique that omits the other substrate(s) may offer insight into ligand-induced structural rearrangements in the active site. Such an approach may be of particular value in tRNA synthetases and other RNA-modifying enzymes, where induced-fit assembly of the active-site conformation upon ligand binding is a common phenomenon (9).

Amino Acid Specificity in GlnRS at the Activation Step—Crystal structures of GlnRS bound in quaternary complexes with tRNA^{Gln}, the nonhydrolyzable ATP analog AMPCPP, and either glutamine or glutamate showed that the enzyme binds glutamate in a distinct nonproductive orientation (6). The nonproductive binding is driven by a new ionic contact with the guanidinium group of Arg-30 that is not made by glutamine. This predicts that a major component of the 107-fold discrimination against glutamate occurs at the first step of aminoclaylation, when the free amino acid binds.

To test this hypothesis we isolated the first step of the reaction using the conventional ATP-PP_i exchange assay. These data showed that the discrimination against glutamate is 4 × 104-fold (Table 1), accounting for a major fraction of the overall 107-fold specificity. In this reaction the discrimination against glutamate arises from at most a 500-fold decrease in \( k_{\text{cat}} \) together with a 100-fold increase in \( K_m \) (Table 1). Although we have not measured the tRNA transfer step independently, it is possible that the rates with which Gln-AMP and Glu-AMP react with the 2'-OH group at A76 of the tRNA will differ less significantly. This is because binding of the activated Gln-AMP and Glu-AMP intermediates could be driven by the common AMP moiety, resulting in a similar orientation of the mixed anhydride linkage relative to the tRNA ribose. In support of this conjecture, a 3.0-Å crystal structure of the GlnRS-tRNA^{Gln} complex bound to a nonhydrolyzable glutamyl adenylate analog\(^2\) shows that the AMP portion of the analog binds nearly identically to the AMP portion of the glutamine analog 5'-O-\([N\-(l\text{-glutaminyl})sulfamoyl]\) adenosine (QSI) (7). The \( k_{\text{cat}} \) for Glu-AMP synthesis (\( k_{\text{cat}} = 0.09 \text{ s}^{-1} \)) is also very similar to the

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\(^2\)E. Franzen, T. Bullock, J. Lapointe, and J. J. Perona, unpublished data.
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$k_{\text{chem}}$ for the overall two-step reaction under the same conditions ($k_{\text{chem}} = 0.06 \text{ s}^{-1}$). Thus, activation of glutamate may be rate-determining for the overall two-step reaction.

Human GlnRS also shows greater amino acid selectivity for overall aminoacylation as compared with the first activation step, although the difference in that case is only 4-fold instead of the $10^2$- to $10^4$-fold for *E. coli* GlnRS (29). This distinction arises from more efficient misaminoacylation by the human enzyme. In *E. coli* GlnRS, the in vitro discrimination factor as assessed from the relative $k_{\text{cat}}/K_m$ values is $4 \times 10^4$-fold, which is substantially higher than the observed overall error rate in protein biosynthesis (~3 in 10,000). However, intracellular concentrations of glutamate in Gram-negative bacteria are generally at least 10-fold higher than those of glutamine (30).

**Amino Acid Discrimination at the Binding and Chemical Steps**—The rapid chemical-quench approach to examine cognate aminoacylation by GlnRS has revealed that the rate-limiting step in the catalytic cycle corresponds to release of aminoacyl-tRNA (15). Thus, the steady-state constants $k_{\text{cat}}$ and $K_m$ are not equivalent to chemical catalysis and substrate binding, respectively. Because of this, an assessment of how amino acid specificity is partitioned at the binding and catalytic steps, and further correlations of the free-energy differences at these steps with crystal structures, necessarily requires the use of transient kinetics. To address this, we employed single-turnover experiments with enzyme in excess of tRNA, at saturating concentrations of ATP and tRNA. The maximal rate of the chemical steps ($k_{\text{chem}}$) is found at saturating levels of amino acid, whereas the binding affinity is derived (for the rapid equilibrium case) by plotting $k_{\text{obs}}$ versus amino acid concentration and fitting to a hyperbolic binding equation. These experiments showed that the glutamate binding site in GlnRS is not saturable even at concentrations up to several molar, so that partitioning of specificity between binding and catalysis can be set only within limits (Table II).

The extremely weak glutamate binding affinity is unexpected, because x-ray crystal structures of the GlnRS-tRNA$^{\text{Gln}}$ complex bound to an ATP analog and either glutamine or glutamate showed apparently stronger binding interactions for the noncognate substrate (6). The side chain of free glutamine binds in the identical position as compared with the enzyme-tRNA complex bound to QSI (6, 7). In both the unliganded and aminoacyl adenylate states, the amide side-chain oxygen of glutamine makes no discriminating contacts with enzyme groups, whereas the NH$_2$ group interacts with the phenolic hydroxyl of Tyr-211 and with a water molecule, each of which are of ambiguous hydrogen-bonding character. By contrast, glutamate binds in a distinct orientation in which one carboxylate oxygen makes an ion-pair contact with the guanidinium moiety of Arg-30, whereas the other oxygen occupies the same position as does -NH$_2$ of glutamine. In this binding mode the α-carboxylate group of glutamate is displaced with respect to the ribose sugar of the 3'-terminal tRNA nucleotide A76. Consequently, it appeared that the rate of the chemical step might be strongly decreased owing to the misorientation of reactive moieties, whereas the binding affinity for glutamate might be strong due to the ion-pair with Arg-30. The relatively weak binding affinity of 1 mM for glutamine is consistent with the absence of strong, orienting polar contacts for the amide group (6).

Discrimination against glutamate at the binding step by GlnRS may arise in part from unfavorable moderate-range electrostatic forces acting to oppose the apparently strong Arg-30 ion-pair. This is suggested based on a comparative analysis of the amino acid binding pockets in GluRS and GlnRS, as revealed by x-ray crystallography and extensive sequence alignments (6, 31, 32). As closely related class Ib tRNA synthetases, GluRS and GlnRS possess topologically identical active-site domains, with the ATP, amino acid, and tRNA 3'-end binding sites in very similar relative orientations. In the structure of the *Thermus thermophilus* GluRS-tRNA$^{\text{Glu}}$ complex bound to glutamate, the substrate side-chain carboxylate group makes three direct ion-pair contacts with two arginine residues conserved among GluRS enzymes (Arg-5 and Arg-205), the first of which corresponds to the strictly conserved Arg-30 in GlnRS (31). Because all GlnRS enzymes lack the second arginine (present at the position corresponding to Cys-229 in *E. coli* GlnRS), the electrostatic potential in the amino acid cleft should be significantly more negative than for GluRS. This is likely a primary mechanism by which GlnRS excludes glutamate; indeed, mutation of the equivalent amino acid in human GlnRS generated a modest increase in the activity of the enzyme toward glutamate (29).

Comparison of ATP-PP$_i$ exchange and overall aminoacylation kinetics also gives some insight into the origins of discrimination against glutamate. At the lower pH of the ATP-PP$_i$ exchange assay (pH 6.0), saturation for glutamate was observed (Fig. 4B). This is in sharp contrast to steady-state and single-turnover experiments for the overall reaction at pH 7.2 or pH 7.4, where no saturation was found (Fig. 4A). This suggests that partial protonation of glutamate at lower pH may render the noncognate substrate more similar to glutamine in its chemical properties. However, the protonation of titratable amino acid side chains in the binding cleft, such as His-215 (Fig. 14) or Cys-229, may provide necessary electrostatic compensation needed for stronger glutamate binding.

Multiple sequence alignments of GlnRS sequences show an accumulation of negatively charged residues in the GlnRS catalytic domain near the amino acid binding cleft (9). Conserved negatively charged groups at positions Glu-34, Glu-73, Asp-212, Glu-222, and Glu-257 of *E. coli* GlnRS are highly conserved among GlnRS across nature; each lies within 10 Å of the amino acid binding site. Structure-based sequence comparisons with GluRS enzymes instead show much less concentration of negative electrostatic potential, as well as the presence of an additional conserved arginine at the position of *E. coli* GlnRS Cys-229. Together, the pH-dependent glutamate saturation

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3. N. T. Uter, I. Gruic-Sovulj, and J. J. Perona, unpublished data.
and predicted conserved differences in binding pocket amino acids argue for a key role for electrostatics in conferring amino acid specificity. Directed protein engineering experiments may provide a convenient approach for testing this hypothesis.

GlnRS enzymes in bacteria owe their existence to a late horizontal transfer from eukaryotes. Because of this, GlnRS and the eukaryotic/archaeal GluRS subfamily are closely related, whereas the other GluRS subfamily representing the bacterial enzymes (such as T. thermophilus GluRS) is more distant (32, 33). The divergence extends to a greater dissimilarity in the predicted local structure of the glutamate binding site, although no crystal structure for a bacterial-type GluRS is yet available.

Amino Acid-specific Affinity for tRNA—The 60-fold improved affinity for tRNA\textsuperscript{Gln} in the presence of cognate glutamine suggests that formation of the tRNA binding site depends on specific contacts made between GlnRS and glutamine in the active site cleft. Although the x-ray structures reveal no direct contact between glutamine and tRNA\textsuperscript{Gln}, it is not difficult to see how the respective binding sites for these substrates may be coupled through the protein structure. The amino acid and tRNA are spatially separated by as little as 6–8 Å, and each makes interactions with the same region of the Rossman fold domain (Fig. 14). One possible pathway that is immediately apparent from inspection of the crystal structures connects glutamine binding to the acceptor stem and 3′-acceptor end of the tRNA. Specific contacts in the minor groove of the acceptor stem at base pair Gly-3 and Cys-70 are mediated through the side chain of Asp-235 emanating from an α-helix in the second half of the Rossmann fold domain (10, 34). Asp-235 is in the direct vicinity of Phe-233, which stacks together with Tyr-211 upon the adenine base of the 3′-terminal A76 nucleotide. Tyr-211, in turn, also accepts a hydrogen-bond from the glutamine substrate side-chain amide.

Numerous prior experiments have also offered evidence that recognition between GlnRS and tRNA\textsuperscript{Gln} is significantly mediated by induced-fit conformational changes in the enzyme and tRNA. First, glutamine affinity depends on contacts of the enzyme with the anticodon nucleotide U35, revealing a long-distance communication pathway between the amino acid and tRNA binding sites (15). Second, fluorescence spectroscopy experiments using GlnRS labeled with an extrinsic probe indicated that both ATP and tRNA binding promotes a conformational change (11). Third, neutron scattering studies have shown significant intensity differences in the high angle region between the unbound and tRNA-bound enzymes, indicative of conformational changes (35). Finally, comparison of x-ray crystal structures in tRNA-bound and unliganded states show that tRNA binding generates structural changes throughout the enzyme, repositioning key active site peptides that bind glutamine and ATP.

An important and distinct feature of the data reported here, however, is the demonstration that the structural linkages between the substrate binding sites may be molecular determinants of specificity. That is, features of the enzyme architecture that control the required ligand-induced conformational changes appear to play a further role in helping to reject noncognate substrates. When glutamate is bound, the association rate of the tRNA is deceased by 100-fold (Table II). This striking effect is not likely to arise from a difference in the rate by which the enzyme and tRNA encounter each other by diffusion, but rather to a sharp decrease in the rate at which the initial “encounter complex” is rearranged into the productive conformation. Further evidence for this proposal is provided by pulse-chase experiments, which demonstrate that an important contribution to specificity arises from the ratio of $k_{\text{off}}$ to $k_{\text{aff}}$, that is, the partitioning between the forward and reverse steps after formation of the Michaelis complex. Although specificity is more commonly achieved by increasing the rate of dissociation for noncognate substrates, here we show that specificity is generated instead by reducing the rate of the chemical step as compared with the rate of dissociation.

GlnRS offers an example of a general phenomenon in enzyme reactions: induced fit provides a means to improve substrate discrimination because noncognate substrates may induce partial or incorrect rearrangements, leading ultimately to misalignment of reactive moieties (effect on the chemical step), or weaker binding affinity for other substrates or cofactors (effects on the binding steps) (36). Thus, the weakened tRNA binding affinity in the presence of glutamate appears to arise from disruption of an intramolecular signaling pathway involving induced-fit rearrangements. Further evidence of discrimination by induced fit is provided by a fluorescence spectroscopy experiment, in which it was shown that noncognate tRNA\textsuperscript{Glu} may not cause the same ATP-dependent conformational change observed when tRNA\textsuperscript{Gln} binds to the enzyme (12).

tRNA-dependent amino acid discrimination has been previously described in the context of amino acid editing reactions for those tRNA synthetases possessing this activity (37). In these reactions, the tRNA dependence involves the synthesis of misacylated tRNA in the common aminoacyl-tRNA synthesis active site, followed by a conformational change of the 3′-acceptor end to redirect the aminoacyl ester linkage into a spatially separate hydrolytic active site. By contrast, the mechanism described here represents an alternative that obviates the need for an editing reaction, providing selectivity that exceeds that of editing synthetases as measured by relative $k_{\text{cat}}/K_v$ values toward structurally related noncognate amino acids (6). The most important challenge in further experiments is likely to be working out, in detail, the nature of the structural pathways mediating the communication between amino acid and tRNA binding sites. The transient kinetics methods described here and elsewhere (15), together with structure-based mutagenesis, provide a viable approach to addressing this question. In turn, knowledge of the pathways of intramolecular communication may be expected to offer significant insights for protein engineering experiments directed toward redesigning specificity and expanding the genetic code.

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