Switching the Amino Acid Specificity of an Aminoacyl-tRNA Synthetase†

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ABSTRACT: The accuracy of protein synthesis essentially rests on aminoacyl-tRNA synthetases that ensure the correct attachment of an amino acid to the cognate tRNA molecule. The selection of the amino acid substrate involves a recognition stage generally followed by a proofreading reaction. Therefore, to change the amino acid specificity of a synthetase in the aminoacylation reaction, it is necessary to alleviate the molecular barriers which contribute its editing function. In an attempt to accommodate a noncognate amino acid into the active site of a synthetase, we chose a pair of closely related enzymes. The current hypothesis designates glutaminyl-tRNA synthetase (GlnRS) as a late component of the protein synthesis machinery, emerging in the eukaryotic lineage by duplication of the gene for glutamyl-tRNA synthetase (GluRS). By introducing GluRS-specific features into the Rossmann dinucleotide-binding domain of human GlnRS, we constructed a mutant GlnRS which preferentially aminoacylates tRNA with glutamate instead of glutamine. Our data suggest that not only the transition state for aminoacyl-AMP formation but also the proofreading site of GlnRS are affected by that mutation.

Aminoacyl-tRNA synthetases catalyze the esterification of tRNAs by the cognate amino acids in a two-step reaction (*1*), leading to the synthesis of an aminoacyl-adenylate intermediate and to the transfer of the activated amino acid on the 3′-terminal adenosine of tRNA:

$$\text{aaRS} + \text{aa} + \text{ATP} \rightleftharpoons \text{aaRS}\cdot\text{aa}\sim\text{AMP} + \text{PP}_i$$

$$\text{aaRS}\cdot\text{aa}\sim\text{AMP} + \text{tRNA} \rightleftharpoons \text{aaRS} + \text{AMP} + \text{aa-tRNA}$$

The selectivity of the overall process implies three major molecular sieves: selection of the cognate amino acid in the active site of the synthetase, discrimination of the corresponding tRNA through a large repertoire of nucleic acid−amino acid interactions, and an editing mechanism devoted to the rejection of misactivated amino acids or misaminoacylated tRNAs (*2*). The latter mechanism has been especially observed in the case of valyl- and isoleucyl-tRNA synthetases, two enzymes which very efficiently activate the isosteric or smaller amino acids threonine and valine, respectively (*3*−*6*). The weak discrimination properties of these enzymes toward the amino acid is compensated by their very ability to reject the misactivated residues in an editing mechanism. The rejection of the misactivated amino acid can be accomplished either before or after its transfer to the cognate tRNA molecule and is usually stimulated following RNA interaction with the synthetase (*2*). Since editing was shown to be an RNA-dependent process (reviewed in ref *7*), it is generally believed that glutaminyl-tRNA synthetase has no hydrolytic editing mechanism. Indeed, although the amino acid activation step can generally be accomplished in the absence of tRNA, a noticeable exception concerns the three class I enzymes arginyl-, glutamyl-, and glutaminyl-tRNA synthetases (*1*). Their strict tRNA-dependent amino acid activation suggested that a proper tRNA−protein interaction induces an essential conformational change that confers a productive conformation on the active site (*8*−*10*).

Among the twenty aminoacyl-tRNA synthetases, glutamyl- and glutaminyl-tRNA synthetases are certainly the most closely related synthetases. It is generally accepted that the genes encoding GluRS[1] and GlnRS are paralogous genes that arose through duplication of an ancestral gene of the GluRS-type and further specialized for two related enzyme activities (*11*). In most of prokaryotic cells, including eubacteria and archaea, a single enzyme of the GluRS-type aminoacylates both $tRNA^{Glu}$ and $tRNA^{Gln}$ with glutamate, the formation of Gln-$tRNA^{Gln}$ proceeding through transamidation of Glu-$tRNA^{Gln}$ (*12*, *13*). A noticeable exception concerns γ purple bacteria which possess a eukaryotic-type GlnRS possibly acquired by horizontal gene transfer (*11*). It was therefore tempting to speculate that the amino acid recognition and editing mechanisms of genuine GluRS and GlnRS significantly diverged to confer on the two daughter enzymes accurate discrimination properties, but retained a conserved general scheme reflecting their common ancestral origin.

In the present study, we have investigated the amino acid selection properties of human GlnRS in the amino acid activation and aminoacylation reactions. On the basis of the phylogenetic analysis of the conserved Rossmann fold domain of the GlxRS (GluRS or GlnRS) sequences (*11*, *12*), and of the crystal structure of *Escherichia coli* GlnRS (*14*) and *Thermus thermophilus* GluRS (*15*), we have introduced by site-directed mutagenesis a limited set of amino acid substitutions into the active site of human GlnRS. The aim

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[1] Abbreviations: GlnRS, glutaminyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase.

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of our approach was to build a mutant human GlnRS with the capacity to aminoacylate tRNA with glutamate instead of glutamine. We report that a double mutation led to a dramatic switch of amino acid specificity from Gln to Glu. This conversion of specificity is mainly accounted for by increased first-order rate constants for amino acid activation and tRNA aminoacylation catalyzed by the mutant GlnRS with glutamate as compared to glutamine.

EXPERIMENTAL PROCEDURES

Expression and Purification of Human GlnRS. For expression of human GlnRS in the yeast *Saccharomyces cerevisiae*, the cDNA encoding its complete coding sequence (*11*) was subcloned into pYeDP60 and then used to transform to Ura^+ and Ade^+ the strain W303-1B α which was grown in rich galactose medium as described (*16*). All purification steps were performed at 4 °C. Cells (15 g) were resuspended in 22 mL of buffer A (10 mM potassium phosphate, pH 7.5; 1 mM EDTA; 10 mM 2-mercaptoethanol; 10% glycerol) and lysed in an Eaton press in the presence of protease inhibitors, and nucleic acids were removed from the clear supernatant by Polymin P (BASF) precipitation. This fraction was applied to a S Sepharose FF column (Pharmacia) developed with a potassium phosphate gradient from 10 to 350 mM. Fractions with GlnRS activity were combined, desalted on Sephadex G-25 column, and applied to a Q Sepharose FF column (Pharmacia) equilibrated in buffer A. The active fractions, eluted in buffer A, were directly applied to a Bio-Gel HTP Hydroxyapatite column (Bio-Rad) equilibrated in buffer B (10 mM potassium phosphate, pH 7.5; 0.01 mM $CaCl_2$; 10 mM 2-mercaptoethanol; 10% glycerol) and developed with a potassium phosphate gradient from 10 to 300 mM. Combined fractions were 2.5-fold diluted and applied to a Macro-Prep Ceramic Hydroxyapatite column (Bio-Rad) developed as above. The purified protein, homogeneous by SDS−PAGE analysis, was stored at −20 °C in 15 mM potassium phosphate pH 7.5, 2 mM dithioerythritol (DTE), 0.1 mM EDTA, and 50% glycerol.

Construction of GlnRS Mutants. The Cys^{456} to Arg mutation was introduced by PCR with primers TCACATATGCGTCTGAC and TGGAATTCCTTGGTGCGGAGTGAGTGAGTG and exchange of the internal *Nde*I-*Eco*RI fragment. The second mutation was generated by PCR with primers ACTCCAGCACAGCCATG and GGGACGTCTATTGCCCTGTGGCTTGGGAGTATGG for the Gln^{481} to Ala mutation or GGGACGTCTATTGCCCTGTGATTTGGGAGTATGG for the Gln^{481} to Ile mutation. Mutated *Aat*II-*Xba*I fragments were exchanged in the GlnRS cDNA. The resulting plasmids were verified by DNA sequencing. The $GlnRS^R$, $GlnRS^{RA}$, and $GlnRS^{RI}$ mutant proteins were purified as described for the wild-type GlnRS. Their elution was monitored by Coomassie Blue staining or Western blotting after SDS−PAGE.

Assay for GlnRS. Initial rates of tRNA aminoacylation were measured at 25 °C in 0.1 mL of 20 mM imidazole−HCl buffer (pH 7.5), 150 mM KCl, 0.5 mM DTE, 5 mM $MgCl_2$, 3 mM ATP, 60 μM (50 Ci/mol) or 2 mM (12.5 Ci/mol), ^{14}C-labeled glutamine or glutamic acid (DuPont NEN), and saturating amounts of tRNA, as previously described (*17*). Beef liver tRNA (glutamine acceptance of 70 pmol/A_{260}) partially purified on a DEAE-Sephadex column (*18*) or total brewer's yeast tRNA (Boehringer, glutamine acceptance of 5 pmol/A_{260}) were used as tRNA substrates. The incubation mixture contained catalytic amounts (0.003−3 μM) of enzymes appropriately diluted in 10 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, containing bovine serum albumin at 4 mg/mL. One unit of activity is the amount of enzyme producing 1 nmol of aminoacyl-$tRNA^{Gln}$/min, at 25 °C. Enzyme concentrations were calculated by using an absorption coefficient of 0.971 A_{280} units cm^2 mg^{-1}. For the determination of K_M values for amino acids in the tRNA aminoacylation reaction, amino acid concentrations of 0.02−3 mM were used. Michaelian parameters were obtained by nonlinear regression of the theoretical Michaelis equation to the experimental curve using the KaleidaGraph 3.0.4 software (Abelbeck Software). The isotopic [^{32}P]PP_i-ATP exchange reaction was assayed at 25 °C in 0.1 mL of 20 mM imidazole−HCl buffer (pH 7.5), 0.1 mM EDTA, 2.5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 2 mM ATP, 2 mM amino acid, 2 mM [^{32}P]PP_i (DuPont NEN, 0.4 or 10 Ci/mol), and saturating amounts of beef or yeast tRNA. The reaction was initiated by the addition of an appropriate enzyme dilution (0.003−3 μM). One unit of activity in the exchange reaction is the amount of enzyme producing 1 nmol of ATP/min, at 25 °C. For the determination of K_M values for the amino acid, concentrations of 0.02−20 mM were used.

RESULTS AND DISCUSSION

A Poorly Aminoacylatable tRNA Fully Activates the ATP-PPi Exchange Reaction. Human GlnRS requires tRNA in the activation step. Its catalytic efficiency in the glutamine activation reaction, expressed as the ratio of the catalytic rate and Michaelis constants k_{cat}/K_M of 4.9×10^5 min^{-1} M^{-1} (Table 1), is reduced to undetectable values in the absence of tRNA. However, the activation and aminoacylation steps are not tightly coupled, and interaction with a competent tRNA molecule rather than its potential to be aminoacylated drives its catalytic site into a conformation suitable for the amino acid activation reaction. Yeast tRNA ($tRNA^Y$) is a poor acceptor tRNA in the aminoacylation reaction catalyzed by the human GlnRS as compared to the cognate beef tRNA ($tRNA^B$) (Figure 1A), with a 200-fold decrease in the k_{cat}/K_M for Gln mainly accounted for by a 31-fold increase in the K_M value for Gln (Table 1). By contrast, yeast tRNA promotes the activation reaction as efficiently as beef tRNA (Figure 1), even with a slight 3-fold increase in the catalytic efficiency. Therefore, a RNA/protein interaction is necessary and sufficient to switch the active site of GlnRS into a productive conformation in the activation reaction with a major positive effect on the apparent binding of Gln, irrespective of the aminoacylation to occur. This result suggested that distinct sites of the catalytic pocket of GlnRS could be involved along the different stages of the overall aminoacylation reaction.

Human GlnRS is Highly Specific for the Amino Acid Gln. The specificity factor, expressed as the ratio of the catalytic efficiencies for glutamine versus glutamate [$(k_{cat}/K_M)^{Gln}/(k_{cat}/K_M)^{Glu}$] in the activation and aminoacylation reactions, in the presence of $tRNA^B$, was estimated to be 30 000 and 105 000, respectively (Table 1). The selectivity of the wild-type human GlnRS for its cognate amino acid glutamine versus glutamate proved to be of the same order of magnitude as

Table 1: Kinetic Constants for Amino Acids Gln and Glu of Wild-Type Human GlnRS in the ATP−PP_i Exchange and TRNA Aminoacylation Reactions, in the Presence of Cognate (Beef, $tRNA^B$) or Uncognate (Yeast, $tRNA^Y$) tRNA[a]

	amino acid activation						tRNA aminoacylation					
	k_{cat}^{Gln} (min^{-1})	k_{cat}^{Glu} (min^{-1})	K_M^{Gln} ($M^{-1} \times 10^3$)	K_M^{Glu} ($M^{-1} \times 10^3$)	k_{cat}/K_M^{Gln} ($min^{-1} M^{-1}$)	k_{cat}/K_M^{Glu} ($min^{-1} M^{-1}$)	k_{cat}^{Gln} (min^{-1})	k_{cat}^{Glu} (min^{-1})	K_M^{Gln} ($M^{-1} \times 10^3$)	K_M^{Glu} ($M^{-1} \times 10^3$)	k_{cat}/K_M^{Gln} ($min^{-1} M^{-1}$)	k_{cat}/K_M^{Glu} ($min^{-1} M^{-1}$)
$tRNA^B$	216 ± 12	0.13 ± 0.03	0.44 ± 0.03	8 ± 3	4.9×10^5	16	27 ± 2	0.03 ± 0.02	0.13 ± 0.02	15 ± 5	2.1×10^5	2
$tRNA^Y$	189 ± 11	nd	0.13 ± 0.02	nd	14.5×10^5		4.2 ± 0.8	ND	4 ± 2	ND	1050	

[a] The homogeneous purified enzyme has a specific activity of 1900 units/mg in the ATP-PP_i exchange reaction (one unit is the amount of enzyme producing 1 nmol of ATP/min at 25 °C) and 290 units/mg in the aminoacylation reaction (one unit is the amount of enzyme producing 1 nmol of aminoacyl-tRNA/min at 25 °C), in the presence of Gln and $tRNA^B$: nd, not determined; ND, Not Detectable.

observed for highly discriminating aminoacyl-tRNA synthetases (*2*). In the activation reaction, the K_M value for the amino acid is increased only 18-fold for Glu (8 mM) as compared to Gln (0.44 mM), but the catalytic rate is severely impaired (0.13 min^{-1} for Glu versus 216 min^{-1} for Gln) (Figure 1 and Table 1). Activation of the non cognate amino acid Glu is also tRNA-dependent and is promoted by yeast tRNA as well (Figure 1B, inset). Therefore, tRNA−protein interaction per se does not provide human GlnRS with unambiguous amino acid discrimination properties. The specificity factor for the amino acid is 4-fold higher in the aminoacylation reaction as compared to the activation step, suggesting that other molecular sieves located beyond the activation step also contribute an essential stage of amino acid selection, even if the former already involves a tRNA-mediated selection. Aminoacylation of yeast tRNA with Glu was not detectable.

Rationale for Site-Directed Mutagenesis of Human GlnRS. A structure-based alignment (*11, 12*) revealed three categories of conserved residues within their catalytic domain. The first category of conserved residues is the GlxRS-specific residues, such as Gly^{274}, His^{277}, or Gly^{279} in the vicinity of the HIGH signature sequence of Class I synthetases, that are encountered in all GluRS and GlnRS. A second category comprises phylogeny-specific residues, that is amino acids that distinguish GlxRSs according to their evolutionary relatedness, not to their aminoacylation specificity. The amino acid sequences of all GlxRSs have been devided into two groups referred to as the Glu-type or Gln-type enzymes (*11, 12*). The Glu-type enzymes include eubacterial GluRSs; the Gln-type enzymes include all GlnRSs as well as GluRSs from archaea and eukarya. In accordance with this classification, residues His^{280} and Val^{493} from human GlnRS are conserved in all GlnRSs and in eukaryotic and archaeal GluRSs but are replaced by Gly^{19} and Lys^{237} in *E. coli* GluRS, a representative of eubacterial GluRSs. The amino acids pertaining to these two classes of conserved residues were excluded from the set of residues potentially involved in the active site of GlxRSs in the selection of the proper amino acid substrate. A third category of conserved residues was identified as function-specific residues. Residues Cys^{456} and Gln^{481} of human GlnRS are conserved in all GlnRSs but are never found in any GluRS. They are replaced by Arg^{199} and Tyr^{224} in *E. coli* GluRS. This third type of conserved residues was selected as a target to change the amino acid specificity of a GlxRS.

Because GlxRS enzymes are closely evolutionary related, we assumed that they share a common structural organization of their catalytic sites amenable to accommodate the amino acids Gln or Glu following a restricted set of point mutations. The challenging perspective was to build a mutant human GlnRS with the capacity to aminoacylate tRNA with glutamate instead of glutamine although retaining its Glx specificity. In a first round of site-directed mutagenesis, the Cys^{456} invariant residue of $GlnRS^{WT}$, located in the amino acid pocket of GlnRS (*14*), was converted to Arg (C456R), the cognate invariant residue for all GluRS, to give $GlnRS^R$. On the basis of the crystal structure of *E. coli* GlnRS (*14*), we observed that a C456R mutation in human GlnRS (Cys^{229} in *E. coli*) could introduce some steric hindrance to the active site. In a second round of mutagenesis, the Gln^{481} residue of $GlnRS^R$ (Gln^{255} in *E. coli*) was replaced by Ile, a residue

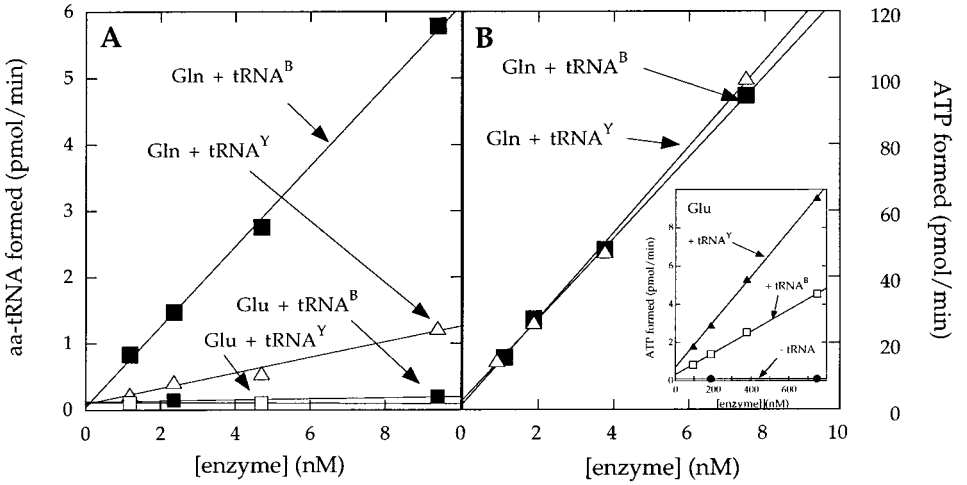


FIGURE 1: Aminoacylation of tRNA (A) and tRNA-dependent amino acid activation (B) for wild-type GlnRS. Initial rates of aminoacylation of beef tRNA ($tRNA^B$) or yeast tRNA ($tRNA^Y$) with the amino acid Gln or Glu by human GlnRS are plotted as a function of enzyme concentration. Activation of the amino acid Gln in the presence of beef or yeast tRNA, or of the amino acid Glu in the absence of tRNA or in the presence of $tRNA^B$ or $tRNA^Y$ (inset) are reported as the amount of ATP formed per minute in the ATP-PP_i exchange reaction. Standard errors on the initial rates are in the range 10−25% of the value.

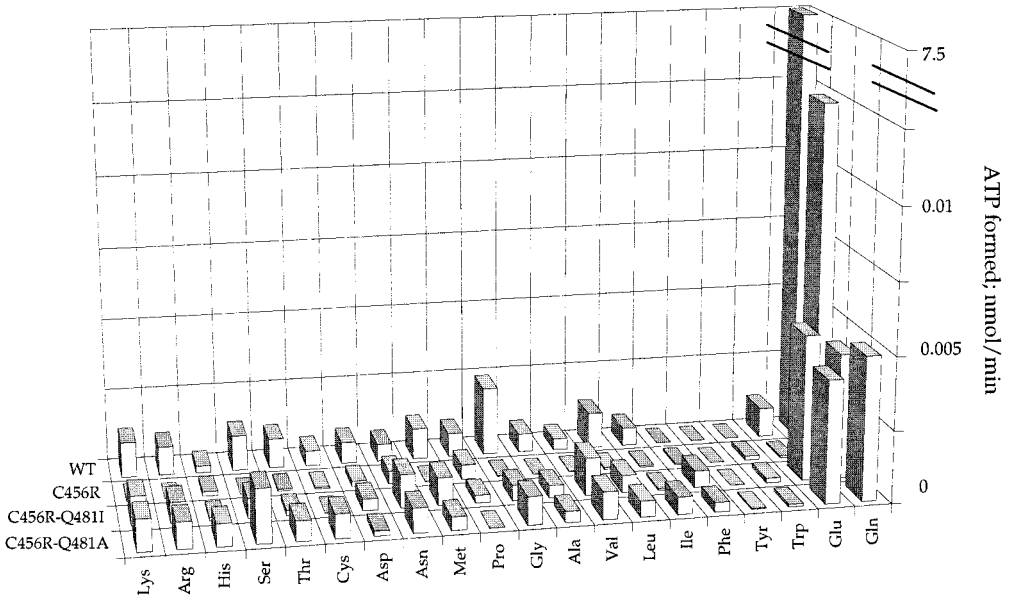


FIGURE 2: Amino acid activation catalyzed by $GlnRS^{WT}$, $GlnRS^{R}$, $GlnRS^{RI}$, and $GlnRS^{RA}$. ATP-PP_i exchange reactions (100 μL) were performed in the presence of 2 mM of each amino acid and 0.4 μM enzyme (8 nM $GlnRS^{WT}$ in the presence of Gln).

found in all eukaryotic GluRS, or Ala, a small hydrophobic residue, to give the double mutants C456R/Q481I ($GlnRS^{RI}$) and C456R/Q481A ($GlnRS^{RA}$), respectively.

Activation of Glutamate by GlnRS. The two double mutants lost their ability to discriminate the amino acid Glu from the natural substrate Gln, but retained their ability to reject all 18 other amino acids (Figure 2). Activation of both amino acids Gln and Glu remains tRNA-dependent. The specificity factor for glutamine versus glutamate in the activation reaction decreased from 30 600 for the wild type to 2.5 for $GlnRS^{RI}$ and 1.2 for $GlnRS^{RA}$ (Table 2). The catalytic efficiency (k_{cat}/K_M) of the mutant enzymes is about 2000-fold decreased in the Gln activation reaction and 6-fold increased in the Glu activation reaction as compared to wild type (Table 2). The rate of Glu activation catalyzed by $GlnRS^{RI}$ is 5-fold that observed in the presence of Gln, as compared to a 1660-fold higher rate for Gln versus Glu catalyzed by $GlnRS^{WT}$. The values for K_M are essentially unchanged: 0.44 and 0.53 mM for Gln, 8 and 7 mM for Glu, respectively, for $GlnRS^{WT}$ and $GlnRS^{RI}$. Consequently, the double mutation confers on GlnRS the ability to catalyze preferentially the activation of the amino acid Glu. Because the K_M of the reaction is still more favorable for Gln versus Glu and catalysis remains tRNA-dependent, we conclude that the main effect of the mutations is to increase the complementarity of the active site for glutamyl-adenylate in the transition state, with the release of a conformational sieve which prevented $GlnRS^{WT}$ from falling into a tRNA-induced active conformation when the amino acid Glu is present in the active site. It is also noticeable that the mutant $GlnRS^{RA}$ displays a 10-fold higher K_M value for Gln as compared to the wild-type GlnRS or the other GlnRS derivatives. This suggests that the amino acid residue at position 481 is also involved to some extent in the initial recognition stage of the amino acid substrate. The substitution of Gln^{481} to Ala contributes +1.6 kcal/mol to the apparent free energy of

Table 2: Kinetic Constants for Amino Acids Gln and Glu of Wild-Type Human GlnRS and of Derivatives Thereof in the ATP−PP_i Exchange and tRNA Aminoacylation Reactions[a]

Amino Acid Activation

	k_{cat}^{Gln} (min^{-1})	k_{cat}^{Glu} (min^{-1})	K_M^{Gln} ($M^{-1} \times 10^3$)	K_M^{Glu} ($M^{-1} \times 10^3$)	k_{cat}/K_M^{Gln} ($min^{-1}\ M^{-1}$)	k_{cat}/K_M^{Glu} ($min^{-1}\ M^{-1}$)	$\frac{(k_{cat}/K_M)^{Gln}}{(k_{cat}/K_M)^{Glu}}$
$GlnRS^{WT}$	216 ± 12	0.13 ± 0.03	0.44 ± 0.03	8 ± 3	4.9×10^5	16	30 600
$GlnRS^{R}$	0.32 ± 0.08	≤0.03	0.36 ± 0.06	ND	890		
$GlnRS^{RI}$	0.14 ± 0.05	0.72 ± 0.08	0.53 ± 0.08	7 ± 3	260	103	2.5
$GlnRS^{RA}$	0.48 ± 0.08	0.48 ± 0.08	7 ± 3	8 ± 3	70	60	1.2

tRNA Aminoacylation

	k_{cat}^{Gln} (min^{-1})	k_{cat}^{Glu} (min^{-1})	K_M^{Gln} ($M^{-1} \times 10^3$)	K_M^{Glu} ($M^{-1} \times 10^3$)	k_{cat}/K_M^{Gln} ($min^{-1}\ M^{-1}$)	k_{cat}/K_M^{Glu} ($min^{-1}\ M^{-1}$)	$\frac{(k_{cat}/K_M)^{Gln}}{(k_{cat}/K_M)^{Glu}}$
$GlnRS^{WT}$	27 ± 2	0.03 ± 0.02	0.13 ± 0.02	15 ± 5	2.1×10^5	2	105 000
$GlnRS^{R}$	0.24 ± 0.05	≤0.01	0.3 ± 0.1	ND	800		
$GlnRS^{RI}$	0.04 ± 0.02	0.51 ± 0.06	0.25 ± 0.05	6 ± 3	160	90	1.8
$GlnRS^{RA}$	ND	ND	ND	ND			

[a] ND: not determinable.

amino acid binding. This less favorable energy of interaction could be due to direct or indirect effects. However, it is conceivable that a contact with the substrate is lost when the side chain of the amino acid at position 481 is shortened.

Switch of Aminoacylation Properties. For the wild-type GlnRS, the specificity factor for Gln versus Glu in the aminoacylation reaction is 4-fold higher than that in the activation reaction (Table 1), thus indicating that the overall amino acid discrimination also involves the second step of the reaction, the aminoacylation step. Although the first step of the reaction, the activation of amino acid, is already tRNA-dependent in the case of GlnRS, a second sieve provides the enzyme with a lower rate of error. This second site of control most certainly also involves tRNA since yeast tRNA promotes activation by wild-type human GlnRS but is poorly aminoacylated, suggesting that different RNA−protein interactions are involved to confer on the catalytic center of the enzyme active conformations in the activation or aminoacylation steps. However, the possibility that nucleotides within the acceptor arm of $tRNA^Y$ act as negative determinants for the proper positioning of that tRNA into the active site crevice cannot be ruled out. Discrimination between Gln and Glu is also relieved at the aminoacylation step for the $GlnRS^{RI}$ and $GlnRS^{RA}$ mutants that display specific activities of 1.5 and 1.2 units/mg for glutamylation of $tRNA^B$, as compared to 290 units/mg for glutaminylation by $GlnRS^{WT}$ (Figure 3). The Michaelis constants in the aminoacylation reaction catalyzed by $GlnRS^{RI}$ determined for glutamine and glutamate showed that the catalytic efficiency (k_{cat}/K_M) of the mutant is 1300-fold reduced for Gln but 45-fold increased for Glu, as compared to $GlnRS^{WT}$ (Table 2). The specificity factor of the mutant is reduced to 1.8. As a control, no glutamylation of $tRNA^Y$ could be detected, thus excluding contamination of $GlnRS^{RI}$ by the endogenous GluRS from the overproducing yeast cell. This result also explains why a human enzyme efficiently misaminoacylating $tRNA^{Gln}$ with Glu is not toxic for yeast. In the case of the mutant $GlnRS^{RA}$, we could not determine the k_{cat} and K_M values for Gln and Glu. Indeed, this enzyme proved to be toxic for the host cell, and high amounts of purified protein could not be obtained. The purified $GlnRS^{RA}$ preparation was able to aminoacylate $tRNA^B$ (specific activity of 1.2 units/mg) and

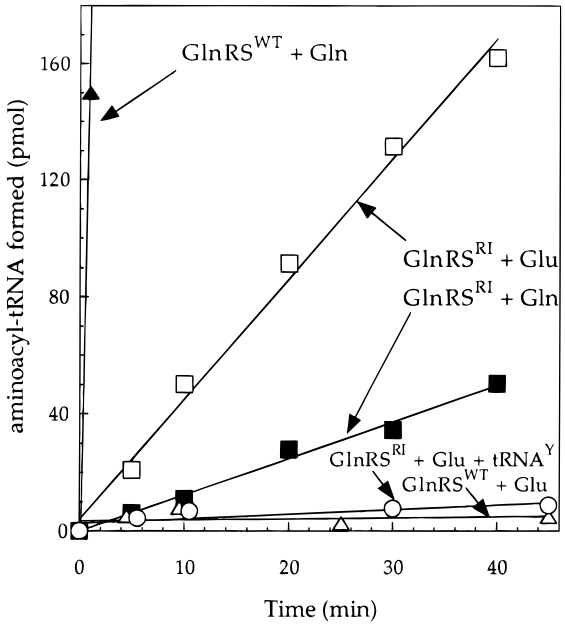
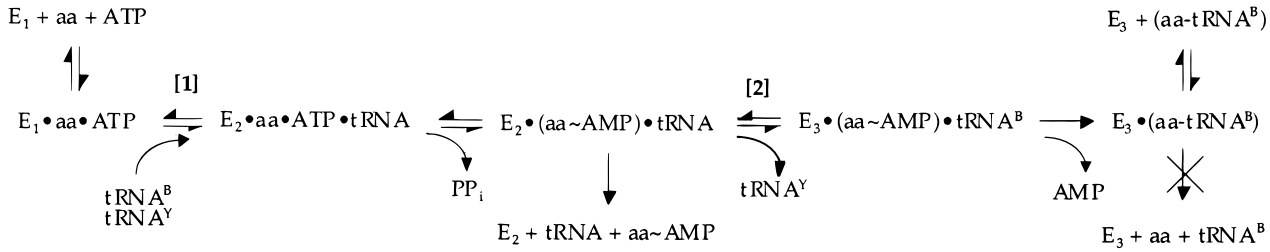


FIGURE 3: Glutamylation of tRNA by $GlnRS^{RI}$. The time courses of aminoacylation of beef tRNA by $GlnRS^{WT}$ or by $GlnRS^{RI}$ with Gln (▲, ■) or Glu (△, □) are compared. As a control, glutamylation of yeast tRNA by $GlnRS^{RI}$ is shown (○). The reaction mixture contained 0.3 μM enzyme, 2.25 mM amino acids, and saturating amounts of tRNA.

$tRNA^Y$ (4.3 units/mg) with Glu. Although the apparent homogeneity of this enzyme preparation is similar to that observed for the other enzyme preparations, as judged by SDS−PAGE analysis, we cannot completely rule out the possibility that it is contaminated by trace amounts of the endogenous yeast GluRS. However, the in vivo toxicity of $GlnRS^{RA}$ suggests that it could significantly misacylate yeast tRNA.

Non-Hydrolysis of aa-tRNA by GlnRS. We were unable to detect any post-transfer editing activity in the case of human $GlnRS^{WT}$ or of the mutant enzyme $GlnRS^{RI}$. When 0.2 μM $tRNA^B$ misacylated with Glu by $GlnRS^{RI}$ is incubated at pH 7.5 in the absence or presence of up to 0.2 μM $GlnRS^{WT}$ or $GlnRS^{RI}$, no enzyme-catalyzed deacylation was observed (not shown).

Scheme 1



CONCLUSION

Our results support Scheme 1, the reaction mechanism for glutaminylation of tRNA by GlnRS. The first key step [1] which converts the inactive form E_1 into an enzyme species E_2 competent in the amino acid activation reaction is an RNA-driven process that can be efficiently triggered by a poorly aminoacylatable tRNA species. The formation of an enzyme species E_3 with an active conformation for the aminoacylation reaction requires both cognate tRNA and aminoacyl-adenylate. The amino acid specificity of the enzyme is contributed by two selection steps: discrimination between Gln and Glu in the activation stage and involvement of the proper aminoacyl-adenylate in promoting the acylation reaction. In the double mutant $GlnRS^{RI}$ the rates of glutamyl-adenylate formation and of transfer of the activated glutamate to tRNA are greatly improved, with little effect on the K_M values for the amino acid. These results suggest that amino acid residues Cys^{456} and Gln^{481} of human GlnRS are not involved in the initial amino acid recognition but play a key role in amino acid selection via the recognition of the aminoacyl∼AMP intermediate of the reaction. Because the catalytic efficiency of the mutant enzyme $GlnRS^{RI}$ is similar for the activation and aminoacylation reactions, as compared to an 8-fold lower k_{cat}/K_M^{Glu} value for $GlnRS^{WT}$ in the aminoacylation reaction relative to the activation step, we conclude that the C456R/Q481I variant has lost its potential to reject glutamate as a misactivated amino acid. In accordance with the scheme of kinetic proofreading (*19*, *20*), if the E_2 to E_3 transition is not allowed, that is when a noncognate aminoacyl-AMP or a noncompetent tRNA is present, the activated aa∼AMP would be released either as such or after hydrolysis of the mixed anhydride bound. Because no post-transfer hydrolytic activity was observed, this step should contribute the major proofreading stage of $GlnRS^{WT}$.

This is the only example of a molecular switch in the amino acid specificity for an aminoacyl-tRNA synthetase. Because of their late duplication from a GluRS-type synthetase after archaea and eukarya diverged, we assume that GlxRSs retain sufficient flexibility in their active site to accommodate a restricted set of "reverse mutations", that is, of mutations susceptible to turn a GlnRS enzyme back to a GluRS enzyme.

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