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Acceptor end binding domain interactions ensure correct aminoacylation of transfer RNA

(tRNA specificity/aminoacyl-tRNA synthetase/nonsense suppression)

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ABSTRACT **The recognition of the acceptor stem of $tRNA^{Gln}$ is an important element ensuring the accuracy of aminoacylation by *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS; EC 6.1.1.18). On the basis of known mutations and the crystal structure of the $tRNA^{Gln}$-GlnRS complex, we mutagenized at saturation two motifs in the acceptor end binding domain of GlnRS. Mutants with lowered tRNA specificity were then selected *in vivo* by suppression of a glutamine-specific amber mutation ($lacZ_{1000}$) with an amber suppressor tRNA derived from $tRNA_1^{Ser}$. The mischarging GlnRS mutants obtained in this way retain the ability to charge $tRNA^{Gln}$, but in addition, they misacylate a number of noncognate amber suppressor tRNAs. The critical residues responsible for specificity are Arg-130 and Glu-131, located in a part of GlnRS that binds the acceptor stem of $tRNA^{Gln}$. On the basis of the spectrum of tRNAs capable of being misacylated by such mutants we propose that, in addition to taking part in productive interactions, the acceptor end binding domain contributes to recognition specificity by rejecting noncognate tRNAs through negative interactions. Analysis of the catalytic properties of one of the mischarging enzymes, GlnRS100 (Arg-130 → Pro, Glu-131 → Asp), indicates that, while the kinetic parameters of the mutant enzyme are not dramatically changed, it binds noncognate $tRNA^{Glu}$ more stably than the wild-type enzyme does (K_d is 1/8 that of the wild type). Thus, the stability of the noncognate complex may be the basis for mischarging *in vivo*.**

Aminoacyl-tRNA synthetases select the correct tRNA substrate among a large number of structurally similar tRNA species with remarkable accuracy (1, 2). A limited set of nucleotides (identity elements) (3) in the tRNA structure have been implicated in this recognition process. Most of these elements are concentrated in the anticodon and the acceptor end region (1), and they are involved in positive interactions, in which direct contacts of the tRNA with the cognate synthetase are made, and negative ones, in which certain features of the tRNA prevent interaction with the noncognate enzymes. While there is much knowledge on the nature of the identity elements in tRNA, our understanding of the specific interactions with the protein is less clear, although crystallographic investigations of tRNA·synthetase complexes (4–7) have recently aided these studies.

The *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS; EC 6.1.1.18) system has been well studied by genetic (8–11), biochemical (reviewed in ref. 12), and biophysical (4, 7, 13, 14) means. This system has the advantage that mutants with reduced tRNA discrimination can be obtained by genetic selection (8), which provides a random approach to search for positions in the enzyme that affect proper tRNA recognition. In this way, GlnRS mutants with relaxed tRNA discrimination (*glnS7*, *glnS10*, and *glnS15*) have been obtained due to amino acid substitutions in domains interacting with the tRNA acceptor stem (14).

We wished to isolate by regional saturation mutagenesis[§] additional *glnS* mutants with relaxed tRNA discrimination based on alterations in two loop motifs of the acceptor end binding domain of GlnRS. Here we describe the isolation of such mutants. On the basis of their properties we propose a model in which these regions are responsible for recognition specificity by rejecting noncognate tRNAs through negative interactions.

MATERIALS AND METHODS

General. Strains BT32, BT63, BT235, and UT172 have been described (15, 16). GlnRS enzymes were overproduced from the appropriate pET3 clones (17) and purified by FPLC/Mono Q (Pharmacia) chromatography.

Plasmids Carrying tRNA Genes. Plasmid pACYC184 was the vector for tRNA genes when double transformation was required. The genes for synthetic amber suppressor tRNAs (ProH, Lys, Ile2, Met, and Val) and *GluA73* (18–20) were excised as a *Pvu* II fragment from pGFIB-1 and recloned in the *Sca* I site of pACYC184. $tRNA^{ProH}$ (19) will be referred to as $tRNA^{Pro}_{(CUA)}$. The *Eco*RI/*Hin*cII and *Eco*RI/*Hin*dIII fragments of the gene for *E. coli* tRNA $^{Ser}_{1(CUA)}$ in M13mp11 (21) were excised and cloned between the *Eco*RI and *Sca* I sites of pACYC184 and in the *Eco*RI/*Hin*dIII sites of pBluescript (Stratagene) to give pACYCtRNA$^{Ser}_{1(CUA)}$ and pBlutRNA$^{Ser}_{1(CUA)}$, respectively.

Plasmids Carrying *glnS* Genes. The 2-kb *Dra* I fragment containing the *glnS* gene with its promoter and terminator (22) was recloned from M13mp9 in pBR322 and pBluescript KS (−), generating plasmids pBRQ6 and pESQ6, respectively. Mutant *glnS* genes (from mutagenesis) were recloned by replacement of an internal *glnS* fragment in pBRQ6. For overexpression, a *Bam*HI fragment (2.3 kb) was inserted into the pET3 transcription vector (17).

Preparation of Mutagenic Oligonucleotides. Two degenerate pools of oligonucleotides were made: Pool 1 contained 50-mers designed to change codons for amino acids 126–138 in helix/loop E of GlnRS, while pool 2 consisted of mismatched 51-mers, corresponding to region 178–188 of loop 1

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Abbreviation: GlnRS, glutaminyl-tRNA synthetase.

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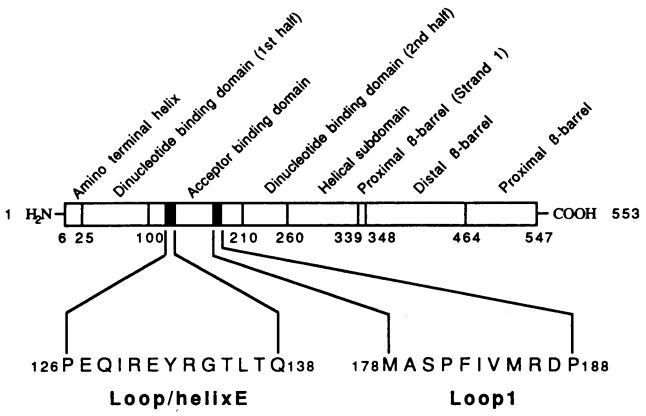
§This treatment may not lead to the isolation of GlnRS proteins in which certain amino acids in the wild-type enzyme are changed to all other 19 amino acids, as *in vivo* stability and toxicity of the mutant enzymes may regulate the viability of the cell.

(see Fig. 1). All members of each mutagenic pool contained one silent mutation designed to introduce a new restriction site: a *Kpn* I site in helix/loop E and a *Cla* I site in loop 1.

Saturation Mutagenesis and Genetic Selection. The procedure for mutagenesis and selection is summarized in Fig. 2. Mutagenesis was performed with a 5:1 molar ratio of synthetic phosphorylated oligonucleotide to U-containing single-stranded pESQ6 DNA (23). The mutagenic mixture was introduced into strain BT235/pACYCtRNA$^{Ser}_{1(CUA)}$ by electroporation. Cells were plated onto M9 salts/lactose agar supplemented with cysteine and ampicillin. Lactose-utilizing colonies were scored after 48-60 hr at 30°C. After plasmid DNA from the positive clones had been screened by retransformation the plasmids were checked for the presence of the new restriction site introduced by the mutagenic primer. The mutational change was determined by DNA sequencing or by PCR using the BRL dsDNA Cycle Sequencing System.

Preparation of tRNAs. Total tRNA was isolated (24) from strain BT235/pBlutRNA$^{Ser}_{1(CUA)}$. Because of its length tRNA$^{Ser}_{1(CUA)}$ was purified by 8% polyacrylamide/urea electrophoresis. Wild-type tRNA^{Gln} (1300), tRNA^{Gln} (1400), and



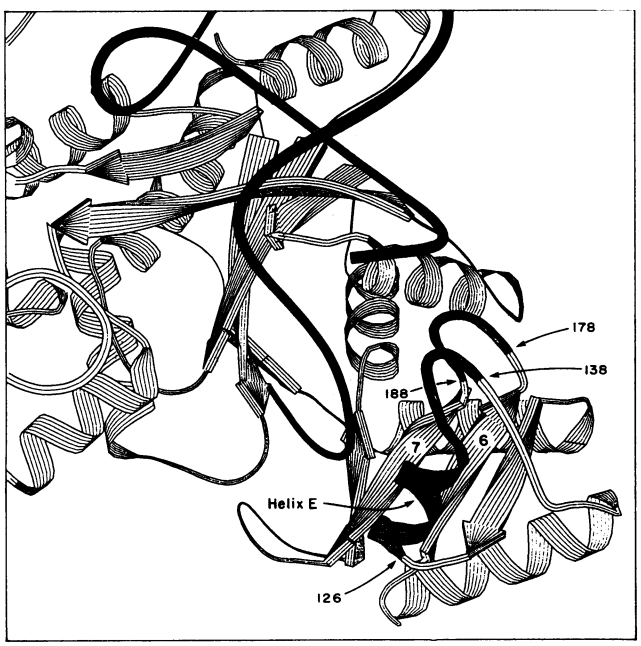


FIG. 1. GlnRS structure. (*Upper*) Arrangement of domains along the GlnRS amino acid sequence. The two mutagenized regions in the acceptor end binding domain (amino acids 126–138 and 178–188) are represented in solid bars. (*Lower*) Ribbon presentation of the acceptor end binding domain of GlnRS interacting with the acceptor stem of tRNA^{Gln} (4). Shaded sections are the two regions whose corresponding DNA sequences were mutagenized. Helix E and strands 6 and 7 are marked. tRNA^{Gln} is indicated in solid black.

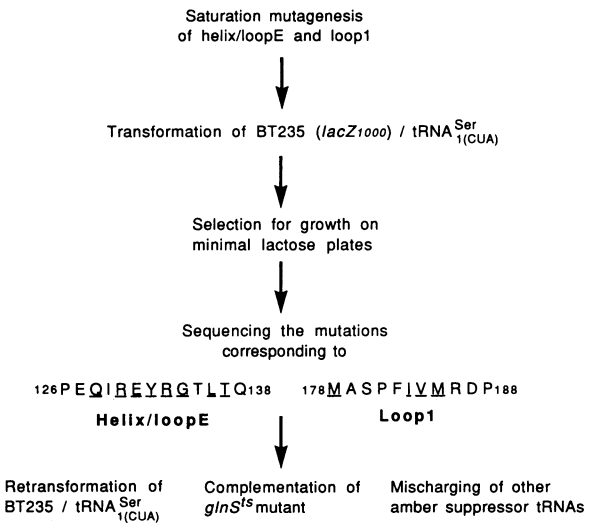


FIG. 2. Scheme for selection of *glnS* mutants.

tRNA$^{Ser}_1$ (1400), with the numbers in parentheses indicating pmol/A_{260} unit, were obtained commercially (Subriden RNA, Rolling Bay, WA).

Determination of Kinetic Parameters. Aminoacylation was done at 37°C as described (25), except that the [^{14}C]glutamine (237 mCi/mmol; 1 Ci = 37 GBq) concentration was increased to 0.3 mM. For tRNA$^{Ser}_{1(CUA)}$ and tRNA^{Glu} charging a concentration range of 10–100 μM tRNA was used. K_d values were determined by nitrocellulose filter binding (26). For noncognate tRNA this was done only with tRNA^{Glu} because the supply of tRNA$^{Ser}_{1(CUA)}$ was limited. The complex of 5′-^{32}P-labeled tRNA and GlnRS was formed by 10-min incubation at 0°C in 40 μl of 50 mM potassium cacodylate, pH 7.0/10 mM magnesium acetate. After dilution with 1 ml of the same buffer the mixture was filtered through a Millipore membrane (0.22 μm), and the filters were dried and their radioactivities were measured. The enzyme and tRNA concentrations were as follows: noncognate complexes, 20 μM enzyme and 4–100 μM tRNA; cognate complexes, 1 μM enzyme and 0.1–10 μM tRNA.

RESULTS

Rationale. The *in vivo* selection for mischarging is based on the *lacZ*$_{1000}$ gene (present in the BT strains), which requires glutamine insertion at the site of the amber mutation to produce active β-galactosidase. This selection has been used to identify positions in GlnRS which, when mutated, give enzymes capable of misacylating the *supF* amber suppressor tRNA^{Tyr} with glutamine (8, 14). One such mutant, *glnS15*, has a single amino acid substitution I129T, located in helix E (Fig. 1). Examination of the GlnRS structure (Fig. 1 *Lower*) made it likely that the extended helix E region (helix/loop E; amino acids 126–138) and the loop 1 (amino acids 178–188) between strands 6 and 7 are involved in tRNA discrimination. Selection for additional *glnS* mutants, which would now permit different noncognate tRNAs to be mischarged with glutamine, might be possible by mutating these regions. The strategy chosen (Fig. 2) for generating the mutants was based on the saturation mutagenesis (randomization of nucleotide sequence at any one position) of those two gene regions with degenerate oligonucleotides and *in vivo* selection of mischarging *glnS* alleles among the mutant population. To enhance possible mischarging and thereby increase the sensitivity of the screen (10) we altered the ratio of synthetase to tRNA by overexpression of the mutant *glnS* and suppressor tRNA genes carried on plasmids (10, 11).

Choice of Suppressor tRNA for Selection Strategy. Two criteria had to be met: the suppressor tRNA should not be glutaminylated by overproduction of wild-type GlnRS *in vivo* (10, 11) and it should have some propensity for being recognized by GlnRS. We thought that the amber suppressor $tRNA^{Ser}_{1(CUA)}$ would be a good candidate, as a mutant form of it was found to be an efficient glutamine-inserting suppressor (21). We then tested this suppressor, together with the others listed in Table 1, for their ability to be glutaminylated *in vivo* by overexpressed wild-type GlnRS (i.e., in a strain with *glnS*$^+$ on a high-copy number plasmid). As Table 1 shows, only *supF* $tRNA^{Tyr}$ could be mischarged under these conditions (10, 11). Thus, we based our selection on growth on minimal lactose medium of strains which would express β-galactosidase by suppression of the *lacZ*$_{1000}$ gene by mischarging of $tRNA^{Ser}_{1(CUA)}$ by a mutant *glnS* allele on a high-copy number plasmid.

Generation and Characterization of Mischarging *glnS* Alleles. Mutagenized pESQ6 DNA was used to transform BT235/pACYC$tRNA^{Ser}_{1(CUA)}$. Among several thousand transformants 8 lactose-utilizing colonies were obtained; plasmid DNA was isolated from these 8 and the regions of site-directed mutagenesis were sequenced. Some of the plasmids proved unstable or had rearrangements in their DNA. We selected 3 clones (*glnS100*, *glnS101*, and *glnS110*) for further characterization (Table 1). In addition, we checked for the appearance of the new restriction site in plasmid DNA samples from 300 transformants without lactose selection. After DNA sequencing, 10 clones with mutations in the desired regions were selected (*glnS102–glnS109*, *glnS111*, and *glnS112*).

To confirm that mischarging of $tRNA^{Ser}_{1(CUA)}$ by the new *glnS* alleles is caused solely by mutations in the acceptor end binding domain, we replaced a 426-bp *Cla* I/*Asu* II fragment encoding this domain in pBRQ6 (carrying *glnS*$^+$) with the corresponding fragment from the *glnS100* gene. The reconstructed *glnS100* in pBR322 suppresses *lacZ*$_{1000}$ in the presence of $tRNA^{Ser}_{1(CUA)}$ and complements the UT172 *glnS*^{ts} allele.

To observe the range of tRNAs that can be mischarged by the mutant *glnS* alleles, we used them to transform strain BT235 carrying the genes for the amber suppressor tRNAs listed in Table 1. The tyrosine and leucine suppressors were contained in strains BT32 and BT63, respectively. It is evident that all *glnS* genes that mischarge $tRNA^{Ser}_{1(CUA)}$ also glutaminylate all other amber suppressor tRNAs tested. However, there are a number of other *glnS* alleles (not selected *in vivo*) that do not charge $tRNA^{Ser}_{1(CUA)}$ but do mischarge some other suppressor tRNAs, most notably $tRNA^{Pro}_{(CUA)}$ and $tRNA^{Met}$. Thus mischarging of $tRNA^{Ser}_{1(CUA)}$ requires different relaxation of discrimination than that of $tRNA^{Pro}_{(CUA)}$.

For the quantitation of mischarging, β-galactosidase activity from the suppression of *lacZ*$_{1000}$ with misacylated Gln-$tRNA^{Ser}_{1(CUA)}$ was determined. As can be seen in Table 2, the GlnRS enzymes with changes in helix/loop E are stronger mischargers than the ones altered in loop 1. In addition, the best mischargers, *glnS100* and *glnS101*, are the ones selected *in vivo*.

To determine whether the mutant GlnRS enzymes still recognize $tRNA^{Gln}$ we tested the complementation of the UT172 *glnS*^{ts} strain (16) with the newly derived *glnS* alleles. As can be seen in Table 1, the *glnS100–glnS111* alleles allow UT172 to grow at 42°C. Interestingly, growth of *glnS* strains that do not mischarge (e.g., *glnS102*) is better, presumably because the presence of the mischarging GlnRS enzymes is detrimental to the cell. The *glnS112* mutant does not complement the *glnS*^{ts} allele. The triple mutation in *glnS112* may lead to a thermolabile GlnRS, to an efficient mischarging enzyme that is toxic to the cell (28), or to a conformational change in the protein resulting in loss of interaction with the identity elements in the acceptor stem of $tRNA^{Gln}$.

Nature of Changes in Mutant *glnS* Alleles. Examination of the sequencing data in Table 1 reveals that strong mischarging enzymes are created when amino acids in helix/loop E in position 130 or 131 are changed, either alone or in combination. For instance, the single amino acid substitutions in *glnS103* (R130H) and *glnS104* (E131D) are sufficient for

Table 1. Characterization of *glnS* mutants

Mutant	Sequence	*glnS*^{ts} compl	Growth by virtue of amber suppressor tRNA mischarged: Ser	Glu-A73	Pro	Lys	Ile2	Met	Val	Leu	Tyr
Helix/loop E, residues 126–138	P E Q I R E Y R G T L T Q										
glnS$^+$		±	−	−	−	−	−	−	−	−	+
glnS100 (C<u>G</u>C → C<u>C</u>C; GA<u>A</u> → GA<u>T</u>)	P D	±	+	+	+	+	+	+	+	+	+
glnS101 (C<u>G</u>C → C<u>A</u>C; G<u>A</u>A → G<u>T</u>A; C<u>G</u>C → C<u>A</u>C)	H V H	±	+	+	+	+	+	+	+	+	+
glnS102 (T<u>A</u>C → T<u>C</u>C; <u>A</u>CG → <u>T</u>CG)	S S	+	−	−	±	−	−	±	−	−	+
glnS103 (C<u>G</u>C → C<u>A</u>C)	H	±	+	+	+	+	+	+	+	+	+
glnS104 (GA<u>A</u> → GA<u>T</u>)	D	±	+	+	+	+	+	+	+	+	+
*glnS105** (G<u>G</u>T → G<u>T</u>T; GA<u>A</u> → GA<u>G</u>)	V	±	+	+	+	+	+	+	+	+	+
glnS106 (C<u>G</u>C → C<u>A</u>C; G<u>A</u>A → G<u>T</u>A)	H V	+	−	−	±	−	−	±	±	−	±
glnS107 (C<u>G</u>C → C<u>A</u>C)	H	+	−	−	±	−	−	±	−	−	±
glnS108 (CA<u>G</u> → CA<u>T</u>)	H	+	−	−	±	−	−	±	±	−	+
glnS109 (<u>C</u>TG → <u>G</u>TG)	V	+	−	−	±	−	−	±	±	−	±
glnS15	T	+	+	+	+	+	+	+	+	+	+
Loop 1, residues 178–188	M A S P F I V M R D P										
glnS110 (ATG → AT<u>A</u>)	I	±	+	+	+	+	+	+	+	+	+
glnS111 (<u>A</u>TG → <u>G</u>TG)	V	±	−	−	−	−	−	±	±	±	+
glnS112 (<u>A</u>TG → <u>A</u>GG; <u>A</u>TC → <u>C</u>TC; <u>G</u>TG → <u>T</u>TG)	R L L	−	+	+	+	+	+	+	+	+	+

Individual mutants identified by *glnS* allele numbers were sequenced (mutations from the wild-type sequence are underlined) and tested overnight for complementation of *glnS172* (*glnS*^{ts}) at 42°C and for the ability to suppress at 30°C by the amber suppressor tRNAs indicated after 3 days on the appropriate lactose minimal plates. Ser denotes $tRNA^{Ser}_{1(CUA)}$. −, No growth; ±, weak growth; +, good growth.
**glnS105* contains a silent mutation.

Table 2. Mischarging by *glnS* mutants

Mutant	% suppression
glnS100	35
glnS101	32
glnS103	26
glnS104	27
glnS105	20
glnS110	14
glnS112	14
glnS$^+$	0.8

β-Galactosidase activity (27) formed by suppression of $lacZ_{1000}$ by Gln-tRNA^{Ser} was used to assay mischarging in strain BT235 grown in M9/lactose. The degree of suppression is expressed as a percentage of the level obtained with *supE* tRNA^{Gln}; 100% equals 435 Miller units (average of four determinations). All mutant *glnS* genes were expressed from pBluescript.

strong misacylation. Crystallographic data (4) show that Arg-130, in conjunction with Ile-129 (the position of change in *glnS15*), contributes to the formation of a "binding pocket" for C-74. Possibly the R130P enzyme has an altered conformation of helix/loop E. The nature of the amino acid substitutions is important: mutations in *glnS106* that alter the same amino acids as in *glnS100* but insert histidine and valine at positions 130 and 131, respectively, do not convert GlnRS into a broad-spectrum mischarging enzyme, while proline and aspartate substitutions do. On the basis of the different properties of the *glnS103* (R130H) and *glnS106* (R130H, E131V) mutants the latter change appears to enhance proper tRNA recognition or compensate for the R130H mutation. Although Arg-133 directly interacts with several sites in the acceptor region of tRNA^{Gln} (14) the His-133 substitution in *glnS107* does not cause general misrecognition of tRNA. However, when this particular change is combined with R130H and E131V a strong mischarging enzyme is made (*glnS101*).

For loop 1 single amino acid replacements with similar characteristics in different positions gave rise the *glnS110* (M185I) and *glnS111* (M178V) mischarging enzymes.

***In Vitro* Characterization of GlnRS100.** To provide biochemical data in support of the *in vivo* results we purified GlnRS100, the best *in vivo* mischarging enzyme, and determined the kinetic parameters in the aminoacylation reaction for mutant and wild-type GlnRS (Table 3). While the K_m value for tRNA^{Gln} is the same for both enzymes, a 3-fold higher k_{cat} value was observed for the wild-type enzyme. The kinetic constants for the glutaminylation of noncognate tRNA^{Glu} and tRNA$_1^{Ser}$ by GlnRS100 are not significantly different from those obtained with wild-type GlnRS (Table 3). However, the higher k_{cat} values observed for tRNA$^{Ser}_{1(CUA)}$ compared with its nonsuppressor parent tRNA$_1^{Ser}$ are probably due to the strong recognition of U-35 in the anticodon of the suppressor tRNA. The differences in the kinetic parameters do not explain the relaxed specificity of the mutant enzymes observed *in vivo*. However, when the binding constants of tRNA·GlnRS complexes were determined by nitrocellulose filter binding and compared to those of wild-type GlnRS, the GlnRS100 protein showed 8-fold tighter binding of noncognate tRNA^{Glu}, while it exhibited binding of the cognate tRNA^{Gln} that was 1/3 as tight. Thus, the binding of the tRNA may be the basis for the mischarging observed *in vivo*.

DISCUSSION

Selection of Mischarging Mutants. A random mutagenesis approach followed by genetic selection has been used previously to isolate GlnRS mutants that are able to glutaminylate *supF* tRNA^{Tyr} (14, 15). The *glnS7*, *glnS10*, and *glnS15* mutations identified two positions (Asp-235 and Ile-129) important for tRNA discrimination by GlnRS. Here we demonstrated that regional saturation mutagenesis followed by *in vivo* selection is a powerful tool for obtaining altered enzymes generating new phenotypes, since a large pool of mutants can be produced and tested for function *in vivo* at once. Although the screen we employed is specific and sensitive, misacylation of tRNA is complicated by additional factors *in vivo*, as competition by cognate and noncognate synthetases is also an element in determining the accuracy of aminoacylation (10, 11). Because mischarged tRNA leads to errors in protein biosynthesis and is therefore detrimental to the cell, the extent of mischarging *in vivo* cannot be too strong and must remain within the limits of cell viability (28, 29). As a result, there is probably a narrow margin in which mutants can be readily selected and maintained. As a matter of fact, the *glnS15* allele obtained *in vivo* (14), which effects misacylation of all the suppressors tested (Table 1), was not picked up in our saturation mutagenesis selection scheme, presumably because its overexpression on pBluescript would kill the cell. The high sensitivity of the $lacZ_{1000}$ suppression assay allows the detection of such mutants, but as a consequence, the effects on their properties *in vitro* may be subtle. The use of amber suppressor tRNAs (with the non-wild-type CUA anticodon) may lead to a bias in the interaction between synthetase and tRNA, but it allows selection specifically for mischarging of amber suppressors.

Location of the Mischarging Mutants in the GlnRS Structure. The predominant identity elements at the acceptor end of tRNA^{Gln}, G-73, G-2·C-71, and G-3·C-70, are important in the synthetase recognition process (4, 12, 14, 25). The enzyme mutants described here are impeded in this intricate mechanism. Whereas the mutations we generated *in vitro* were randomly distributed along the target sequence, the subset of mutations that confer mischarging is centered on residues Arg-130 and Glu-131 in helix/loop E and Ile-183, Val-184, and Met-185 in loop 1. These residues are part of two finger-like structures which, by penetrating the minor groove of the acceptor stem, appear to play an important role in tRNA selection (14). Hence, it was proposed that only tRNA^{Gln} can adopt a sequence-specific conformation complementary to the protein surface in this part of the acceptor end binding domain. Arg-130 and Glu-131 are located in the middle of helix E, which is followed by a β-turn containing Leu-136, whose side chain is wedged between A-72 and G-2·C-71. Amino acid changes in positions 130 (*glnS100*) or 131 (*glnS100* and *glnS104*) may distort helix E, possibly leading to the mispositioning of Leu-136. Therefore, alter-

Table 3. Kinetic constants of wild-type GlnRS and GlnRS100 with different tRNAs

	Wild-type GlnRS				GlnRS100			
tRNA	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m (relative)	K_d, μM	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m (relative)	K_d, μM
tRNA^{Gln}	0.5	2.3	1	0.05	0.5	0.76	1	0.17
tRNA^{Glu}	59	2.7×10^{-3}	10^{-5}	17.3	105	2.7×10^{-3}	1.8×10^{-5}	2.08
tRNA$^{Ser}_{1(CUA)}$	46	0.13	6.1×10^{-4}	ND	25	2.9×10^{-2}	7.9×10^{-4}	ND
tRNA$_1^{Ser}$	38	3.5×10^{-3}	2.0×10^{-5}	ND	38	1.9×10^{-3}	3.2×10^{-5}	ND

ND, not determined.

ation of the accurate positioning of helix E could lead to mischarging by disturbing the process by which the first base pair of the tRNA is recognized. A conservative replacement in this position, L136V (*glnS109*), gives rise to a weak mischarging phenotype; thus, the nature of the side chain is important for accurate tRNA discrimination.

The proper placement of the acceptor stem is crucial for accurate tRNA discrimination by GlnRS. The GlnRS·$tRNA^{Gln}$ structure shows (4) that Arg-133 interacts at several sites near the acceptor end of $tRNA^{Gln}$: a ribose hydroxyl of A-72 and the phosphates of G-73 and C-74. Consequently an amino acid change in position 133 leads to mischarging (*glnS101* and *glnS107*).

In the second finger-like β-turn (loop 1), Ile-183 normally interacts with a water molecule and the G-2·C-71 base pair to form complementary hydrogen-bonding surfaces between the protein and tRNA. In the mischarging mutants *glnS110* and *glnS112* residues at the tip of the finger are affected (M185I and I183L, respectively), possibly altering this particular conformation.

Comparison of *in Vivo* and *in Vitro* Situations. This study again underscores the exquisite sensitivity of the *in vivo* selection for relaxed tRNA discrimination through suppression of $lacZ_{1000}$ by glutaminylated noncognate suppressor tRNA species. Although this is a complex system, the observed lactose-utilizing phenotype is very distinct. In contrast, the observed effect on the catalytic properties of the mutant enzymes in the much simpler *in vitro* system (the only macromolecules present are enzyme and tRNA) is much more subtle, possibly because of limitations in the dynamic range of the assay or because of structural compensation/relaxation of the protein during assay or purification.

What Causes Mischarging? Accurate aminoacylation depends on productive interactions of certain functional groups of the protein with complementary elements in the tRNA. The *in vivo* situation requires additional precision, as "negative elements" on the enzyme preclude the proper positioning of noncognate tRNAs and thus prevent acylation. The most surprising result in this work is that the mutants selected to mischarge $tRNA^{Ser}_{1(CUA)}$ also mischarged our set (eight in total) of other amber suppressor tRNAs tested. Thus, this group of *glnS* mutants exhibits a more general loss of discrimination against noncognate tRNAs. However, the randomly picked mutants in helix/loop E (*glnS106–glnS109*) mischarge only a subset of the test set, the tRNAs derived from $tRNA^{Pro}$, $tRNA^{Met}$, and $tRNA^{Val}$. Therefore, the mischarging spectrum of the selected mutant depends on the nature of the tRNA used in the screen. Given this criterion, it appears that $tRNA^{Ser}_{1(CUA)}$ is less "related" to $tRNA^{Gln}$ than some other amber suppressor tRNAs are. It may be relevant that $tRNA^{Pro}_{(CUA)}$ shares more bases considered identity elements (12) with $tRNA^{Gln}$ than does $tRNA^{Ser}_{1(CUA)}$.

GlnRS is composed of four domains, one being the catalytic core and the others being involved in tRNA selection. Since our mutants exhibit a more general loss of tRNA specificity we propose that the mutations eliminate features of "negative elements" from the protein rather than adding specific interaction with other tRNA substrates. Thus, a role of the acceptor end binding domain of GlnRS may be to prevent the formation of productive interactions with noncognate tRNAs. This is in agreement with the measured GlnRS·tRNA dissociation constants, as the mutant GlnRS100 enzyme exhibited 8-fold tighter binding (compared with wild-type GlnRS) of noncognate tRNA while showing 1/3 as tight binding of the cognate substrate. It is interesting to consider the energetics of this process; stability of the cognate complexes ($K_d \approx 50$ nM) is only 50–100 times better than that of the noncognate complexes (30, 31). Thus, there is considerable energy of interaction (presumably ionic) with a small contribution of energy from specific interactions (32). Hence, an 8-fold tighter association with noncognate tRNA coupled to an increased cellular concentration would be sufficient to cause mischarging *in vivo*. In a more general context, it is interesting to consider that protein specificity is brought about, together with direct recognition, by negative interactions with noncognate substrates.

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