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Transfer RNA mischarging mediated by a mutant *Escherichia coli* glutaminyl-tRNA synthetase

(aminoacyl tRNA synthetase/tRNA specificity/nonsense suppression)

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ABSTRACT **We have isolated mutations in the *Escherichia coli glnS* gene encoding glutaminyl-tRNA synthetase [GlnS; L-glutamine:tRNA^{Gln} ligase (AMP-forming), EC 6.1.1.18] that give rise to gene products with altered specificity for tRNA and are designated "mischarging" enzymes. These were produced by nitrosoguanine mutagenesis of the *glnS* gene carried on a transducing phage (λp*glnS*$^+$). We then selected for mischarging of *su*$^+$*3* tRNA^{Tyr} with glutamine by requiring suppression of a glutamine-requiring β-galactosidase amber mutation (*lacZ*$_{1000}$). Three independently isolated mutants (*glnS7*, *glnS8*, and *glnS9*) were characterized by genetic and biochemical means. The enzymes encoded by *glnS7*, *glnS8*, and *glnS9* appear to be highly selective for *su*$^+$*3* tRNA^{Tyr}, because *in vivo* mischarging of other amber suppressor tRNAs was not detected. The GlnS mutants described here retain their capacity to correctly aminoacylate tRNA^{Gln}. All three independently isolated mutant genes encode proteins with isoelectric points that differ from those of the wild-type enzyme but are identical to each other. This suggests that only a single site in the enzyme structure is altered to give the observed mischarging properties. *In vitro* aminoacylation reactions with purified GlnS7 protein show that this enzyme can also mischarge some tRNA species lacking the amber anticodon. This is an example of mischarging phenotype conferred by a mutation in an aminoacyl-tRNA synthetase gene; the results are discussed in the context of earlier genetic studies with mutant tRNAs.**

It is well established that the translation of genetic information during protein biosynthesis proceeds with a high degree of precision (1, 2). The frequency of errors in selecting between two chemically related amino acids in protein synthesis was experimentally determined to be ≈3/10,000 (3). The high specificity of aminoacyl-tRNA synthetases for their amino acid substrates contributes significantly to the low rate of translational errors (4, 5). In contrast, the capacity of these enzymes to discriminate between the cognate and noncognate tRNAs is much less than that observed for amino acid substrates. Steady-state K_m values for correct and incorrect tRNAs differ for the average aminoacyl-tRNA synthetase by a factor of only 100 (1).

Genetic studies in a number of laboratories demonstrated over a decade ago that the *Escherichia coli* glutaminyl-tRNA synthetase [GlnS; L-glutamine:tRNA^{Gln} ligase (AMP-forming), EC 6.1.1.18] exhibits such a weak discrimination between tRNAs. Mutations in tRNA genes were found whose products could be misaminoacylated or "mischarged" with glutamine by GlnS. For example, a change in tRNA^{Trp} to the *su*$^+$*7* amber suppressor by a substitution at the central base of its anticodon causes it to be mischarged with glutamine (6, 7) (Fig. 1). In the case of *su*$^+$*3* tRNA^{Tyr}, the anticodon mutation generating the suppressor tRNA is insufficient to promote mischarging; however, mischarging by GlnS does occur when additional single base mutations in the 3′-terminal region of the acceptor stem are present (11–15) (Fig. 1). Despite an extensive search for tRNA mutants that are mischarged by other activating enzymes (8, 13), GlnS remains the only aminoacyl-tRNA synthetase conclusively shown to make mischarging errors *in vivo*.

Studies examining the molecular nature of the tRNA binding domain of aminoacyl-tRNA synthetases have been severely hindered by the limited available sequence and structure information on this enzyme family. We recently cloned the structural gene for GlnS (*glnS*) (16) and determined its complete primary sequence (17, 18). In light of the proven role of GlnS in mischarging suppressor tRNA mutants, it seems reasonable to expect that mutations could be obtained in the enzyme that would decrease its specificity for tRNAs. The availability of GlnS mischarging mutants will provide a direct approach to characterize the structural features of an aminoacyl-tRNA synthetase that guides its specificity in tRNA recognition. The mutants should also be useful in efforts to unravel the enzymatic mechanism of GlnS. In this paper, we describe the genetic and biochemical approaches used to isolate and characterize a class of *glnS* mutants that causes the mischarging of *su*$^+$*3* tRNA^{Tyr} as well as certain wild-type tRNAs with glutamine.

MATERIALS AND METHODS

Bacteria and Phages. *E. coli* strain BT1000 (19) was used to construct different suppressor-containing strains. Its genotype is F$^-$ *lacZ*$_{1000}$ *trp*$_{am}$ *str*$^-$ *su*$^-$ (19). Strain BT32 was generated by P1 transduction of *su*$^+$*3* (*supF*) into BT1000. The other amber suppressor strains containing *su*$^+$*1* (Ser), *su*$^+$*2* (Gln), and *su*$^+$*6* (Leu) were constructed in the same way. Strain AB4143 containing a thermolabile GlnS (*glnS1*) is described in ref. 20. The origins of strain N3-1 (*trp*$^-$ *gal*$^-$ *uvrB*$^-$ *str*^{r}) (21) and of λ*susP*$_{am3}$ (22) and T4$_{amC266}$ (22) are given in the papers referenced.

Genetic Manipulations. Procedures for bacterial cell growth, phage infection, P1-mediated transduction, and induction of lysogenized bacteria are described by Miller (23). Nitrosoguanidine mutagenesis was according to ref. 24.

Phage-Encoded Protein Synthesis. *E. coli* strain N3-1, lysogenic for λ*imm*^{21} *ind*$^-$ (21), was grown in M9 medium supplemented with tryptophan (25 μg/ml). Cells in logarithmic phase were irradiated with UV light in a Petri dish with an incident dose of ≈6600 erg/mm^2, centrifuged, resuspended in 20 mM $MgCl_2$ at a concentration of 10^9 cells per ml, and infected with the different λp*glnS* transducing phages (moi, 25). Infected cells (2×10^8) were resuspended in 1 ml of the original medium and labeled with 20 μCi of [^{35}S]methionine (600 Ci/mmol; 1 Ci = 37 GBq) at 37°C for 30 min. The cells were centrifuged at 4°C, resuspended in 50 μl of lysis buffer

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Abbreviation: GlnS, glutaminyl-tRNA synthetase.

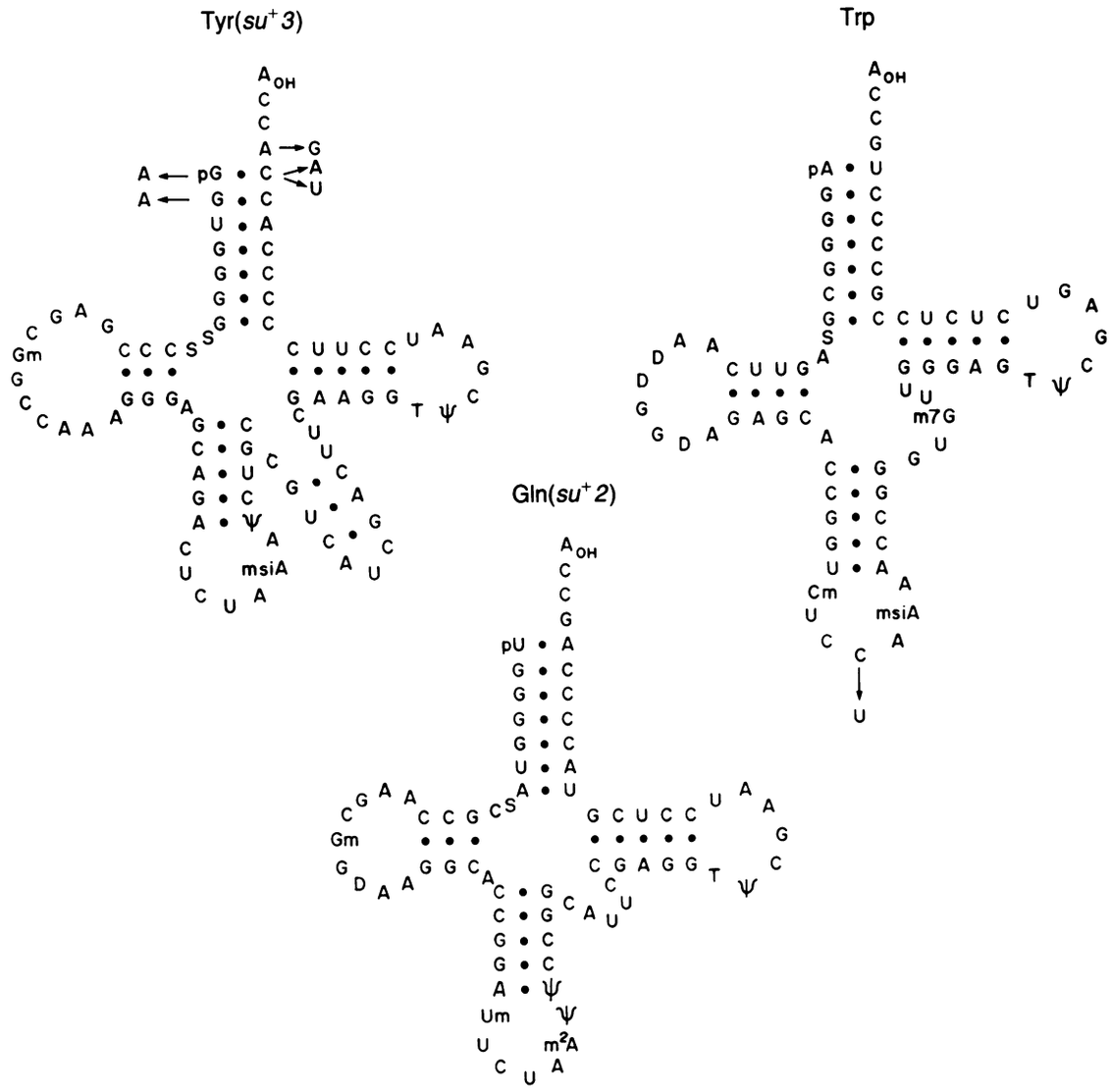

FIG. 1. Structures of *E. coli su*$^+$*3* tRNA^{Tyr}, *su*$^+$*2* tRNA^{Gln}, and tRNA^{Trp} indicating positions of mutations that result in mischarging by GlnS$^+$. Mutations in the tRNA^{Tyr} and tRNA^{Trp} sequences known to result in mischarging by wild-type GlnS are indicated. The arrows indicate the nucleotide substitutions in certain positions of tRNA^{Tyr} and tRNA^{Trp} that allow these tRNAs to be mischarged with glutamine. Each substitution alone is sufficient to cause mischarging (reviewed in refs. 8–10).

A (25) and lysed by several cycles of freezing and thawing. Samples (10 μl) were loaded on an isoelectric focusing slab gel (14 × 14 × 0.1 cm) and run as described (25).

Enzyme Purification. Wild-type GlnS was purified from *E. coli* strain JA221 harboring the cloned *glnS* gene as described (17). The mutant enzyme (GlnS7) was prepared from *E. coli* stain HB101 carrying *glnS7* on plasmid pBR322 (18). Modification of the purification scheme originally designed for the wild-type enzyme was unnecessary for preparing the mutant enzyme.

Table 1. Mischarging by *glnS7* allows suppression

E. coli strain	Lactose utilization: Growth on plates	Lactose utilization: β-Galactosidase activity	Phage growth: λ*susP*$_{am3}$	Phage growth: T4$_{amC266}$
lacZ$_{1000}$ *su*$^-$	−	0.1	−	−
lacZ$_{1000}$ *su*$^+$*2*	+	100	+	+
lacZ$_{1000}$ *su*$^+$*3*	−	0.1	−	−
lacZ$_{1000}$ *su*$^+$*3* (λp*glnS*$^+$)	−	0.1	−	−
lacZ$_{1000}$ *su*$^+$*3* (λp*glnS7*)	+	20	+	+
lacZ$_{1000}$ *su*$^-$ (λp*glnS7*)	−	0.1	−	−

β-Galactosidase activity was measured 30 min after enzyme induction at 37°C (27) and is given relative to a 100% value in the *lacZ*$_{1000}$ *su*$^+$*2* strain. Suppression of λ*susP*$_{am3}$ or T4$_{amC266}$ was tested by spotting the amber mutant phage (about 10^9 per ml) on lawns of strains with or without λp*glnS*$^+$, λp*glnS7*, λp*glnS8*, or λp*glnS9* lysogens. The phage immunity of λp*glnS* is imm^{21} and that of λ*susP*$_{am3}$ is imm$^{\lambda}$. The results obtained with λp*glnS7* are also representative of those with λp*glnS8* and λp*glnS9*.

Table 2. *In vivo* determination of *glnS7*-mediated mischarging of various amber suppressor tRNAs

Transducing phage	*E. coli* $lacZ_{1000}$ carrying			
	su^+1 (Ser)	su^+2 (Gln)	su^+3 (Tyr)	su^+6 (Leu)
None	−	+	−	−
λp*glnS*$^+$	−	+	−	−
λp*glnS7*	−	+	+	−

Growth of *E. coli* $lacZ_{1000}$ strains carrying the indicated amber suppressors was tested on minimal-lactose plates by cross-streaking with transducing phages carrying either *glnS*$^+$ or *glnS7*. The results obtained with λp*glnS7* are also representative of those with λp*glnS8* and λp*glnS9*.

tRNA Isolation. Unfractionated tRNA was prepared from BT32 cells by phenol extractions followed by chromatography of the aqueous phase on DEAE-cellulose as described (26). Crude MRE600 tRNA was purchased.

RESULTS

Isolation of GlnS Mischarging Mutants. The effect of nonsense mutations at certain amino acid positions in a polypeptide chain can be reversed only by suppressor tRNAs that insert an amino acid compatible with the function of the gene product. For example, a nonsense mutation in β-galactosidase ($lacZ_{1000}$) can be suppressed by glutamine (su^+2 $tRNA^{Gln}$) insertion but not by tyrosine (su^+3 $tRNA^{Tyr}$) insertion (27). Strains harboring this mutation are thus phenotypically Lac$^-$ in the absence of a glutamine-inserting suppressor tRNA. We took advantage of this fact to isolate mischarging mutations in the *glnS* gene that were capable of mischarging the su^+3 $tRNA^{Tyr}$ amber suppressor, thus allowing suppression of the $lacZ_{1000}$ mutation.

E. coli strain BT32 ($lacZ_{1000}$, su^+3) was infected with nitrosoguanidine-mutagenized λp*glnS*$^+$ phage, then plated on minimal-lactose medium for the selection of phenotypically Lac$^+$ colonies. After a 2-day incubation at 37°C, the resulting colonies were picked and purified. Prophages were induced by mitomycin c, the lysates were cross-streaked on strain BT32, and Lac$^+$ colonies were selected. Mutant phages that gave Lac$^+$ transductants upon cross-streaking were then purified from single plaques. Three of seven independent phage isolates (λp*glnS7*, λp*glnS8*, and λp*glnS9*) were analyzed in detail.

Since the suppression efficiencies of different amber codons vary, we wanted to know whether amber mutations other than $lacZ_{1000}$ would function in our selection procedure. Therefore, we examined the suppression of two other amber mutations in the λp*glnS7*, λp*glnS8*, and λp*glnS9* lysogens. As was true for $lacZ_{1000}$, $\lambda susP_{am3}$ and $T4_{amC266}$ are both suppressed by su^+2 but not by su^+3 (Table 1). These results indicate that su^+3 $tRNA^{Tyr}$ cannot suppress these glutamine-requiring amber mutations in the absence of the mutant *glnS* genes. Since we observed suppression of all three mutations, we conclude that the *in vivo* level of mischarging in the λp*glnS7* strains is sufficient for more than a single type of amber mutation.

Specificity of *in Vivo* Mischarging Suppression by *glnS7*. Genetic studies with tRNA mutants having altered amino acid acceptor specificity implicated wild-type GlnS in the mischarging of both su^+3 $tRNA^{Tyr}$ and su^+7 $tRNA^{Trp}$ species (6, 7). We wanted to determine whether the mischarging capacity of the mutant enzyme is restricted to these amber suppressor tRNAs. Table 2 shows the results of *in vivo* assays for mischarging suppression of the $lacZ_{1000}$ mutation in the presence of su^+1 (Ser), su^+2 (Gln), su^+3 (Tyr), or su^+6 (Leu) tRNAs. Mischarging of the su^+1 or su^+6 suppressor tRNAs was not detected in the λp*glnS7*, λp*glnS8*, or λp*glnS9* lysogens; thus, the mutant enzyme does not exhibit a general broadening of specificity.

Since a wild-type copy of the *glnS* gene was present in all of these lysogens, to test the specificity of the *glnS7* gene product for the cognate $tRNA^{Gln}$ we used the temperature-sensitive *E. coli* strain AB4143 (20) carrying the gene for a thermolabile GlnS. The λp*glnS7* prophage was able to confer temperature resistance to this strain (data not shown), indicating that the mischarging enzyme retains its ability to aminoacylate $tRNA^{Gln}$ *in vivo*.

The Isoelectric Points of Wild-Type and Mutant GlnS Differ. In an effort to verify that the observed mischarging phenotype of the λp*glnS7*, λp*glnS8*, and λp*glnS9* lysogens was a direct result of a mutation in the *glnS* gene, we examined the phage-encoded *glnS* gene products for al-

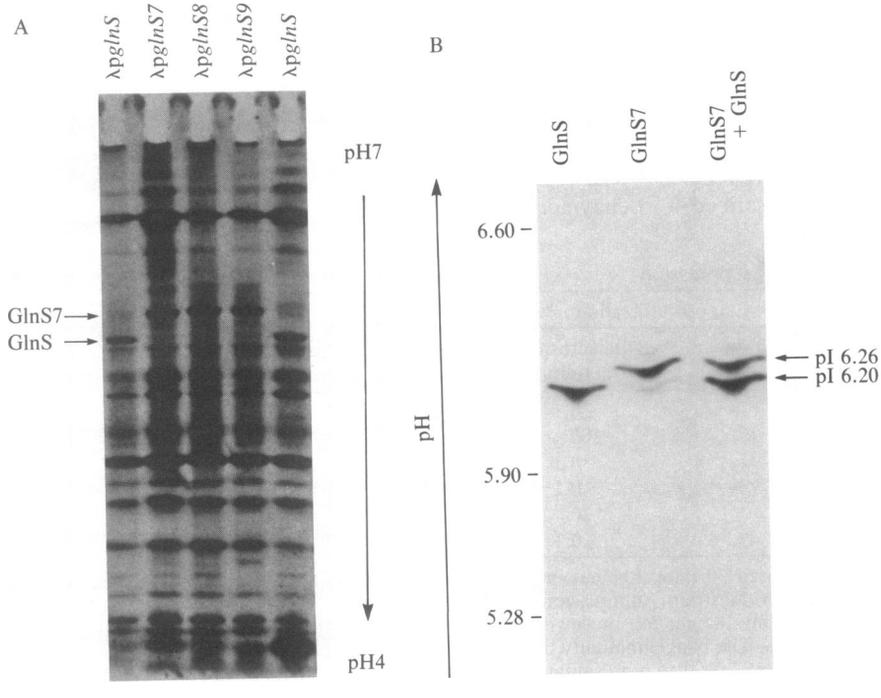

FIG. 2. Isoelectric focusing of GlnS proteins. (*A*) UV-irradiated *E. coli* N3-1 cells were infected with the various *glnS* transducing phages and then labeled with [^{35}S]methionine for 30 min at 37°C. Cell extracts were run on a denaturing isoelectric focusing gel. (*B*) Purified wild-type (GlnS$^+$) and mutant (GlnS7) proteins were resolved by isoelectric focusing in a denaturing polyacrylamide slab gel (15 × 17 × 0.15 cm) (25). The gel was stained with Coomassie blue. Ten micrograms of each designated protein was used. Arrows denote the isoelectric points of the enzymes. The pH at the indicated positions was determined by excising fragments along the length of the gel and measuring the pH of each after suspending it in distilled water.

tered behavior in isoelectric focusing gel electrophoresis. Phage-encoded proteins were specifically labeled with [^{35}S]methionine upon infection of a UV-irradiated *E. coli* strain with the three mutant λp*glnS* phage isolates or with λp*glnS*$^+$. Fig. 2*A* shows an autoradiogram of a urea/polyacrylamide isoelectric focusing gel comparing labeled GlnS proteins in extracts obtained from each of the infected strains. The positions of bands corresponding to the mutant GlnS proteins from each of the three phage isolates are indistinguishable from each other, but they migrate further toward the basic end of the pH gradient than the wild-type enzyme. The mutant and wild-type proteins are identical in $NaDodSO_4$/polyacrylamide gel analysis (data not shown). The apparent change in the isoelectric point of the mutant enzymes is consistent with our expectation that the mischarging phenotype of the λp*glnS7*, λp*glnS8*, and λp*glnS9* lysogens is due to an alteration in the GlnS structure and suggests that this particular type of functional change is restricted to a particular position in the polypeptide chain. This apparent site specificity does not seem to be due to the use of nitrosoguanidine in mutagenesis, because we have also isolated spontaneous λp*glnS7* mutants by an identical selection scheme. Strains infected with these phages had suppression patterns indistinguishable from the mutant phage obtained by chemical mutagenesis (data not shown); however, the isoelectric points of protein from the spontaneous mutants were not analyzed. The frequency of spontaneous mutation from λ*glnS*$^+$ to λp*glnS7* was $\approx 10^{-7}$, consistent with a single nucleotide substitution mutation.

***In Vitro* Mischarging Properties of GlnS7.** Analysis of the tRNA specificity of these GlnS mischarging mutants is limited *in vivo* to suppressor tRNAs. We purified the protein encoded by the λp*glnS7* phage to examine its mischarging potential *in vitro*. For purification, we used an HB101 *E. coli* strain carrying the *glnS7* gene on plasmid pBR322 (18). Although the host copy of the wild-type *glnS* gene is still present in this strain, the plasmid-encoded *glnS7* gene product is overproduced 7- to 10-fold, as measured by titration of cell extracts with GlnS-specific antibodies (20). Fig. 2*B* shows the purified GlnS$^+$ and GlnS7 enzymes, as distinguishable by their mobilities on a denaturing isoelectric focusing polyacrylamide gel.

We used unfractionated tRNA preparations to determine which tRNAs can be mischarged by GlnS7 *in vitro*. Under the condition generally used for *in vitro* aminoacylation assays, the tRNA substrate is present in molar excess over the enzyme, making it impossible to show misacylation. We thus varied the enzyme/tRNA ratio in the assay mixture until an increase in the level of glutamine incorporation (i.e., above that representing the amount of $tRNA^{Gln}$ in the sample) was observed (data not shown). Mixed tRNA preparations with or without *su*$^+$*3* $tRNA^{Tyr}$ were analyzed for glutamine incorporation. For each tRNA sample, the time course of aminoacylation was measured in the presence of either the wild-type or the mutant enzyme (Fig. 3). A high level of GlnS7 was required under these aminoacylation conditions for maximum glutamine incorporation. Fig. 3 *A* and *B* shows that when the amount of *su*$^+$*3* $tRNA^{Tyr}$ in the tRNA preparation is increased (tyrosine acceptance in the phage-induced tRNA is ≈3 times greater than in the uninduced sample), acylation with glutamine increases proportionally. This indicates that this molecule is specifically mischarged by GlnS7 *in vitro*. In contrast, an excess of wild-type enzyme does not significantly mischarge *su*$^+$*3* $tRNA^{Tyr}$ in these experiments.

Mischarging with glutamine of $tRNA^{Tyr}$ appears to be predominantly to the suppressor species, because the considerable amount of wild-type $tRNA^{Tyr}$ present in unfractionated tRNA of strains BT32 and MRE600 (tyrosine acceptance is 30 and 45 pmol/A_{260} unit, respectively) is not

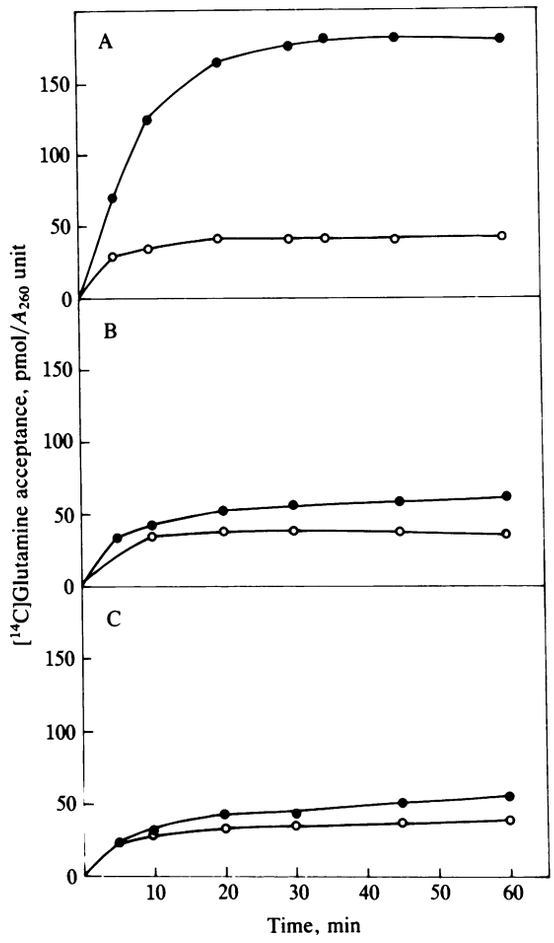

FIG. 3. *In vitro* aminoacylation of unfractionated tRNA with purified wild-type and mutant GlnS. GlnS-catalyzed glutamine-tRNA formation was measured by aminoacylation in a reaction mixture containing 100 mM sodium cacodylate, pH 7.0/10 mM magnesium acetate/2 mM ATP/10 μM [^{14}C]glutamine (specific activity, 250 Ci/mol), 3 A_{260} units of unfractionated tRNA per ml and 50 units of GlnS$^+$ (○) or GlnS7 (●) per ml (17). Prior to addition to the reaction mixture, enzymes were always diluted in a buffer containing 100 mM sodium cacodylate, pH 7.0/20 mM 2-mercaptoethanol/1 mg of bovine serum albumin per ml/50% (vol/vol) glycerol. After incubation at 37°C for the indicated times, 60-μl aliquots were assayed for acid precipitable radioactivity by the filter paper method (28). Tyrosine acceptance was measured with a crude S-100 preparation. (*A*) Unfractionated tRNA with high levels of *su*$^+$*3* $tRNA^{Tyr}$ was isolated from strain BT32 infected with φ80*su*$^+$*3* as described (29). Tyrosine acceptance was 89 pmol/A_{260} unit. (*B*) Unfractionated tRNA from BT32 cells (*su*$^+$*3*). Tyrosine acceptance was 30 pmol/A_{260} unit. (*C*) Unfractionated tRNA from *E. coli* MRE600 (*su*$^-$). Tyrosine acceptance was 45 pmol/A_{260} unit.

significantly mischarged by GlnS7 (Fig. 3 *B* and *C*). The fact that the anticodon mutation in *su*$^+$*7* $tRNA^{Trp}$ is sufficient to allow mischarging with glutamine by wild-type GlnS *in vitro* (9), and that double mutants of $tRNA^{Tyr}$ (i.e., those with alterations in both the anticodon and the amino acid acceptor stem) can also be misacylated in assays using large amounts of this enzyme (13), implies that the anticodon sequence of tRNA may be an important structural feature in the recognition by GlnS. We were thus surprised at the result shown in Fig. 3*C*, which indicates that GlnS7 is able to misacylate some wild-type tRNAs, albeit at low levels.

DISCUSSION

Single-base substitutions in tRNAs that result in altered amino acid acceptor specificity have been termed "mischarg-

ing'' mutations (8). Fig. 1 shows the sequences and predicted secondary structures of $tRNA^{Gln}$, $tRNA^{Tyr}$, and $tRNA^{Trp}$, indicating the positions of tRNA mutations that result in mischarging suppression of nonsense codons. In this study, we have taken advantage of the known role of GlnS in the mischarging of these genetically altered tRNAs to isolate an example of a mischarging aminoacyl-tRNA synthetase mutant. In choosing *su*$^+$*3* $tRNA^{Tyr}$ as a mischarging substrate, we hoped to bias the outcome of our selection scheme toward the isolation of GlnS mutants defective in discriminating between cognate and noncognate tRNA acceptor stem regions.

Using nitrosoguanidine mutagenized λp*glnS* transducing phages, we isolated several independent GlnS mutants whose presence as lysogens in *lacZ*$_{1000}$ *su*$^+$*3* strains resulted in mischarging suppression. The *su*$^+$*3* gene used in our selection had none of the acceptor stem mutations previously shown to cause mischarging by wild-type GlnS. Using *in vivo* suppression with glutamine as an assay, we showed that all of the λp*glnS7* mutants characterized misacylate *su*$^+$*3* $tRNA^{Tyr}$ but not other amber suppressor tRNAs tested. Although it is important to emphasize that a low level of mischarging might not be detectable by our genetic analysis, these results are consistent with the observation that previously identified glutamine-specific mischarging mutants were restricted to tryptophan and tyrosine amber suppressor tRNAs (reviewed in refs. 8–10). Our *in vivo* assay only detects glutamine-mischarging of suppressor tRNAs; however, the slow growth rate of λ*glnS7* lysogens is consistent with possible *in vivo* mischarging of wild-type tRNAs.

All three *glnS7*-, *glnS8*-, and *glnS9*-encoded proteins have an identical isoelectric point that is slightly more basic than that of wild-type GlnS. The observed phenotypes of these mutants may thus arise from the same amino acid change; this hypothesis will be tested by determining the exact molecular nature of each *glnS* mutation. Since aminoacyl-tRNA synthetases bind cognate and noncognate tRNAs with similar affinities, these enzymes must share some general recognition features, with charging specificity achieved by only a few unique interactions (1, 2). Thus, it is reasonable to expect that only one or a few unique alterations could lead to a GlnS structure with the observed mischarging properties.

Was our selection limiting because we identified only one type of mischarging mutation? If the selection required too high a level of mischarged tRNAs, then the cell may not be viable. However, as β-galactosidase mutants have been successfully used to select inefficient nonsense suppressors, we reasoned that even a low concentration of functional enzyme is sufficient for cell growth on lactose medium. Therefore, high levels of mischarging are not needed to suppress the *lacZ*$_{1000}$ mutation.

Our *in vitro* aminoacylation experiments using purified GlnS7 show that its highest affinity appears to be for *su*$^+$*3* $tRNA^{Tyr}$; however, wild-type tRNAs can also act as substrates for this mutant enzyme. It seems unlikely that the observed increase in glutamine incorporation over that required to charge completely the available $tRNA^{Gln}$ is due to generalized mischarging. Indeed, preliminary experiments aimed at identifying which tRNAs are mischargeable reveal that only a subset is aminoacylated with glutamine; these can be separated from glutamine-$tRNA^{Gln}$ and uncharged tRNAs by chromatographic methods (unpublished results). Possible candidates for mischarging might be the set of tRNAs with uridine in the middle position of their anticodons, since the anticodon (30) and especially this site (9) is thought to be important for recognition.

The identification of mischarged species of tRNA should allow us to define further a family of tRNAs that can associate with both their cognate activating enzyme and GlnS. Determination of the amino acid change (or changes) in the mischarging mutants will allow us to begin defining functional sites of GlnS.

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