Nucleic Acids Research, Vol. 20, No. 11 **2847–2852**

Competition of aminoacyl-tRNA synthetases for tRNA ensures the accuracy of aminoacylation

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Received January 17, 1992; Revised and Accepted March 12, 1992

ABSTRACT

The accuracy of protein biosynthesis rests on the high fidelity with which aminoacyl-tRNA synthetases discriminate between tRNAs. Correct aminoacylation depends not only on identity elements (nucleotides in certain positions) in tRNA (1), but also on competition between different synthetases for a given tRNA (2). Here we describe *in vivo* and *in vitro* experiments which demonstrate how variations in the levels of synthetases and tRNA affect the accuracy of aminoacylation. We show *in vivo* that concurrent overexpression of *Escherichia coli* tyrosyl-tRNA synthetase abolishes misacylation of *supF* $tRNA^{Tyr}$ with glutamine *in vivo* by overproduced glutaminyl-tRNA synthetase. In an *in vitro* competition assay, we have confirmed that the overproduction mischarging phenomenon observed *in vivo* is due to competition between the synthetases at the level of aminoacylation. Likewise, we have been able to examine the role competition plays in the identity of a non-suppressor tRNA of ambiguous identity, $tRNA^{Glu}$. Finally, with this assay, we show that the identity of a tRNA and the accuracy with which it is recognized depend on the relative affinities of the synthetases for the tRNA. The *in vitro* competition assay represents a general method of obtaining qualitative information on tRNA identity in a competitive environment (usually only found *in vivo*) during a defined step in protein biosynthesis, aminoacylation. In addition, we show that the discriminator base (position 73) and the first base of the anticodon are important for recognition by *E. coli* tyrosyl-tRNA synthetase.

INTRODUCTION

The fidelity of the translation step in protein biosynthesis is dependent on the ability of an aminoacyl-tRNA synthetase to discriminate with remarkable selectivity amongst many structurally similar tRNAs. At least two factors are important in determining the accuracy of aminoacylation: (i) 'identity elements' in tRNA denote nucleotides in certain positions crucial for positive interactions with the protein or negative interactions preventing non-cognate association (reviewed in 1), and (ii) the occurrence *in vivo* of competition between aminoacyl-tRNA synthetases for a particular tRNA which may have ambiguous identity (reviewed in 3).

Genetic (1,4–6), biochemical (7–10) and biophysical approaches (11–13) are being employed to determine the nature of tRNA identity for many acceptor RNAs chiefly in *E. coli*. For the tRNAs used in this study, glutamine (14) and tyrosine (15, reviewed in 16) identity elements have been shown to be located in the anticodon, the discriminator base in position 73 (17), the acceptor helix (for glutamine) and in the extra arm (for tyrosine).

Transfer RNA molecules are structurally very similar and have affinity for both cognate and non-cognate synthetases (*e.g.*, 18,19). As expected, competition between aminoacyl-tRNA synthetases has been shown to be crucial to the accuracy of protein biosynthesis, presumably by affecting the accuracy of the aminoacylation process directly (2,20–22). This phenomenon may be a particularly important factor in the aminoacylation of tRNAs with ambiguous identity (2,22). All conclusions about tRNA identity derived from *in vivo* approaches already include the effect of competition (1,6,23–25) although for detailed studies variations in concentrations of tRNA and synthetases should be made (22). There are however several limitations to the *in vivo* systems as tRNA identity can only be studied in a suppressor background: in the case of amber suppressors, there is an inherent bias toward recognition by GlnRS and LysRS (reviewed in 1), while in the case of the missense suppression initiation assays (23–27), 'initiation competence' is required for translation. Furthermore, the results of the *in vivo* assays reflect the participation of the tRNAs in all the steps of protein biosynthesis, not just aminoacyl-tRNA formation. Finally, the *in vivo* assays are extremely sensitive. Although attempts have been made to correlate the kinetic parameters obtained *in vitro* with the results of *in vivo* Dihydrofolate Reductase (DHFR) experiments (22), the competition phenomenon has not been directly examined by *in vitro* aminoacylation using purified components.

Here we describe an *in vitro* competition system which allows us to assess the effect of competition on the accuracy of aminoacylation of *in vivo* made suppressor as well as non-suppressor tRNAs. The assay also permits the examination of the relative affinities of various synthetases for a given tRNA.

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The competition phenomenon appears to be particularly relevant for the *E. coli* GlnRS system which we have chosen to study. Not only is the *in vivo* ratio of GlnRS:tRNA^{Gln} approximately 1:1 (28), but also it has been demonstrated that the level of GlnRS influences the accuracy of translation *in vivo* (2). The tRNAs chosen for these experiments (Fig. 1) are *supF* tRNA^{Tyr} which was shown to be mischarged by GlnRS when GlnRS was overproduced (2) and tRNA^{Glu}, as the amber suppressor derived from tRNA^{Glu} was shown to be mischarged *in vivo* by glutamine (reviewed in 1).

MATERIALS AND METHODS

General

Purified *E. coli* tRNAs (with the specific acceptor activity) were purchased from Subriden RNA in the case of tRNA^{lu} (1450pmol/A_{260}) and tRNA^{Tyr} (1500pmol/A_{260}), while tRNA^{ln} (1500pmol/A_{260}) was prepared as described (29). Highly purified *E. coli* aminoacyl-tRNA synthetases (of the specific activity) were obtained as follows: GlnRS (886 nmol/mg·min) was purified as described (30); GluRS (1005 nmol/mg·min) and MetRS were the gifts of U. Michelsen and R. Basavappa, respectively. [^{14}C]Amino acids and enzymes used for cloning and mutagenesis were obtained commercially. Aminoacyl-tRNA synthetase purifications were carried out at 4°C unless otherwise noted. Media for bacterial growth were standard (31).

Strains and plasmids

E. coli strains used in this study include: BT32 (32) (F$^-$, $lacZ_{1000}$, met_{am3}, trp_{am}, str^-, tsx_{am}, bfc_{am}, *supF*), BL21(DE3) (33), CJ236 and a λ^+ derivative of DH1. The plasmids used for overproducing GlnRS and TyrRS, pRS11 and pBRTyrTS (gift of D. Barker), respectively, have been described previously (2,34). Plasmid pJA11 (gift of J. Arnez) is derived from pUC19 and contains an *Eco*RI-*Bam*HI fragment with an artificial *supF* tRNA^{Tyr}G73 gene constructed with the T7 promoter positioned such that transcription starts with G1 of the mature tRNA. The *supF* tRNA^{Tyr}A73 gene (in plasmid pJS7, a construct analogous to pJA11) was obtained by site-directed mutagenesis (35) from *supF* tRNA^{Tyr}G73.

Preparation of supF tRNA^{Tyr}A73 and supF tRNA^{Tyr}G73 by *in vivo* overexpression

E. coli strain BL21(DE3) was transformed with the plasmid pJS7 or pJA11. Induction of T7 RNA polymerase was as described (33) with the exception that ampicillin was boosted at the time of the IPTG induction. The cells (from 750 ml cultures) were collected by centrifugation and resuspended in 10 ml of 10 mM magnesium acetate, 10 mM Tris, pH 7.5. An equal volume of water-saturated phenol was added and the mixture shaken gently at room temperature for 20 min. After a low speed centrifugation, the aqueous phase was removed and the phenol layer re-extracted with TE, pH 7.5. The aqueous phases from the two extractions were then combined, re-extracted with phenol and then ethanol precipitated. The RNA was resuspended in 10 ml Tris, pH 9.0 and incubated at 37°C for 30 min to deacylate the tRNA. The deacylated RNA was re-precipitated and resuspended in water. After a low-speed spin (to remove any insoluble material) the supernatant was applied to 25 ml DEAE-cellulose which had been pre-equilibrated with 0.2 M NaCl. Bound tRNA was eluted with 1.0 M NaCl and ethanol precipitated. Approximately 2 mg of crude tRNA was loaded on a 40 cm×20 cm×1 mm polyacrylamide (8%) gel containing 7M urea and the electrophoresis carried out until the xylene cyanol marker had traveled 30 cm. This gave good separation of short extra arm tRNAs from those with long extra arms. The *supF* tRNA^{Tyr} containing band was excised and eluted (in 0.5 M Tris, pH 7.0, 0.1% SDS, 0.1 mM EDTA, 1 mM $MgCl_2$) by shaking at room temperature after a rapid freeze-thaw. The eluent was then passed over a G-50 column. Levels of overexpression of *supF* obtained ranged from 30–40% of total tRNA following induction. When the *supF* tRNA^{Tyr}G73 preparation was charged *in vitro* with tyrosine a plateau of 151 pmol/A_{260} was reached very quickly. As nucleotide changes in position 73 are detrimental to tyrosylation (see below) we estimate that there is a maximum of 10% wild-type tRNA^{Tyr} contamination.

Purification of TyrRS

E. coli strain DH1λ^+/pBRTyrTS was grown in LB medium containing ampicillin (0.1 mg/ml) to an $A_{600} \approx 1.0$. The ampicillin was boosted halfway through the growth. The cells were harvested and washed with buffer A (20 mM potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol). The cells were re-pelleted, resuspended in a total of 90 ml of buffer A and then disrupted by sonication. The mixture was centrifuged at 15,000 g for 30 min and the resulting supernatant was centrifuged for 1 hr at 100,000 g. The final supernatant (S-100) was dialyzed against buffer A containing 10% glycerol and then applied to a 50 ml DEAE-cellulose column (pre-equilibrated with buffer A). After elution with a 0–250 mM KCl gradient in buffer A, the fractions which contained TyrRS activity were pooled and dialyzed against buffer B (20 mM potassium phosphate, pH 7.8, 10 mM 2-mercaptoethanol) containing 20% glycerol. This fraction was loaded on a 50 ml DEAE-A50 Sephadex column (pre-equilibrated in buffer B) and eluted with a 100–250 mM KCl gradient in buffer B. The fractions with TyrRS activity were pooled and dialyzed against buffer C (10 mM KCl, 20 mM potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol). The pooled sample was then loaded on a hydroxyapatite Ultrogel FPLC column and eluted at room temperature with a gradient of 60–180 mM potassium phosphate, pH 7.0. This column separated TyrRS activity from the remaining GlnRS activity. The TyrRS preparation appeared as one band (0.03 μg applied) on a silver-stained SDS-PAGE gel and had a specific activity of 1467 nmol/mg·min.

In vivo competition assays

E. coli strain BT32 was transformed first with pRS11 (a pACYC184 derivative carrying the gene encoding GlnRS) and then with either pBRTyrTS (a pBR322 derivative containing the gene encoding TyrRS) or with pBR322. The strain BT32/pRS11/pBRTyrTS grows very slowly. Due to the instability of this strain, it was necessary to maintain double antibiotic selection to prevent segregation (observed on MacConkey lactose plates). Four individual transformants of both BT32/pRS11/pBRTyrTS and BT32/pRS11/pBR322 were used to inoculate minimal A medium supplemented with ampicillin (5.0 μg/ml), chloramphenicol (3.4 μg/ml) and casamino acids (0.2%). The cultures were grown overnight at 37°C and diluted 1:10 into fresh medium supplemented as above and containing 1 mM IPTG (t=0). The cell densities of all of the cultures at the time of induction (t=0) were comparable. At two hr intervals, a 1.0 ml sample was removed for A_{600} determination and then split into duplicate 0.5 ml samples. These were added to 0.5 ml

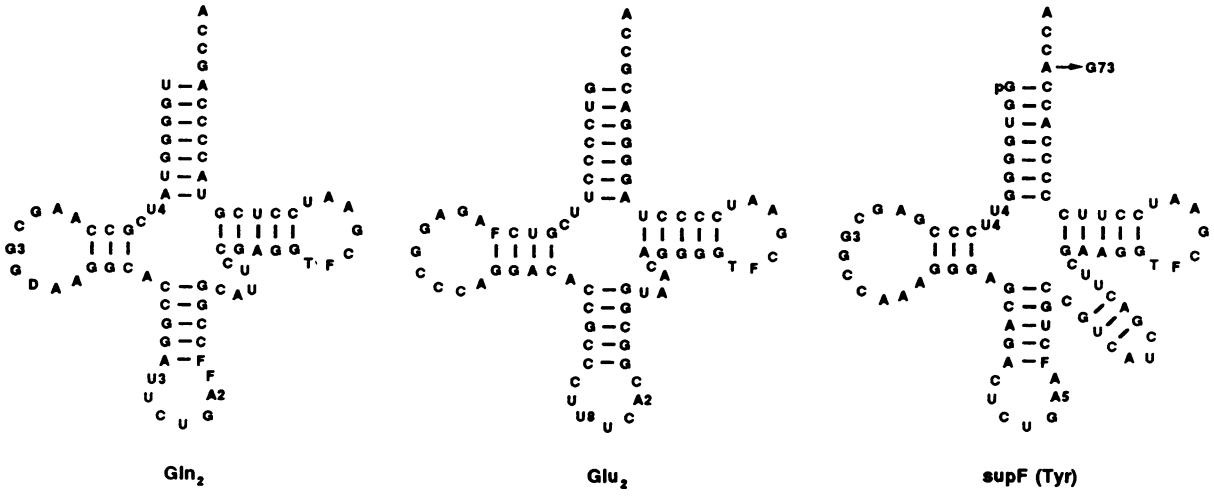

Figure 1. Cloverleaf representations of the sequences of $tRNA^{Iu}$, $tRNA^{In}$ and *supF*$tRNA^{Tyr}$ with the mutation A→G73.

Z buffer for β-galactosidase assays. Each point (in Fig. 2) is an average of four independent isolates and differences in A_{600} were taken into account in calculating the β-galactosidase units (31). The level of TyrRS overproduction was assayed by comparing TyrRS activity in mini-protein preparations (36) of strains BT32/pRS11/pBRTyrTS and BT32/pRS11/pBR322.

Aminoacylation assays

Reaction mixtures (60 μl) contained 100 mM sodium cacodylate, pH 7.0, 10 mM magnesium acetate, 2–4 mM ATP, and [^{14}C]amino acid (specific activities varied from 22–720 cpm/pmol). In all cases, ATP and the amino acid were at or near saturating concentrations. For the *in vitro* competition experiments, cold amino acid specific for the competing synthetase was added at saturating or near saturating concentrations (600 μM Glu and 120 μM Tyr). Enzymes were diluted in enzyme dilution buffer (50 mM sodium cacodylate, pH 7.0, 20 mM 2-mercaptoethanol, 10% (v/v) glycerol and 1mg/ml bovine serum albumin) and the amount added did not exceed 10% of the reaction volume. Incubation was at 37°C for the appropriate times. The reaction mixture or an aliquot was spotted onto 3MM filters (in case of charging with [^{14}C]Tyr the filters were pre-soaked in 1.0–2.5 mM [^{12}C]Tyr to prevent non-specific binding of the radioactive free amino acid), washed in 5% TCA several times, soaked in 95% ethanol, dried and analyzed by liquid scintillation counting.

RESULTS

In vivo competition

We wished to provide further support for the idea that the accuracy of aminoacylation *in vivo* is critically dependent on the relative concentrations of tRNAs and their cognate aminoacyl-tRNA synthetases (2,21,22) and that the amount of free, uncomplexed tRNA or enzyme is crucial for tRNA to be misacylated. For the *in vivo* studies in *E. coli*, we employed a system which takes advantage of the $lacZ_{1000}$ amber mutation in the β-galactosidase gene; active enzyme can be formed by glutamine but not by tyrosine insertion at the site of the amber mutation. Thus, strain BT32 ($lacZ_{1000}$, *supF*) is phenotypically Lac$^-$, unless *supF* $tRNA^{Tyr}$ is misacylated with glutamine (32). Earlier experiments had shown that *supF* $tRNA^{Tyr}$ was mischarged by GlnRS when this enzyme was overproduced and that this effect could be abolished when the cognate substrate, $tRNA^{Gln}$, was overexpressed concurrently (2). We then asked if overproduction mischarging of *supF* $tRNA^{Tyr}$ by GlnRS could be abolished if TyrRS was concurrently overproduced, as in this case *supF* $tRNA^{Tyr}$ would be complexed with TyrRS.

The experiment made use of two strains; BT32/pRS11/pBRTyrTS and BT32/pRS11/pBR322. The first strain contains two plasmids overexpressing independently GlnRS (pRS11) and TyrRS (pBRTyrTS). The latter strain overexpresses only GlnRS. The results (Fig. 2, top) demonstrate that when GlnRS alone is overproduced, β-galactosidase activity, and thus the amount of mischarging, increases with cell density. However, concurrent overproduction of TyrRS gives rise to a much slower growing strain (note the difference in the growth curves for Fig. 2 top and bottom) and effectively abolishes mischarging of *supF* $tRNA^{Tyr}$ Fig. 2, bottom). The β-galactosidase activity, which is approximately the same in both strains at the beginning of the experiment, is about 80-fold lower in the strain overproducing both GlnRS and TyrRS after 8 hr (see different scales in top and bottom panel of Fig. 2) and actually decreased with increasing cell density. Assays of the mini-protein extracts (36) from these strains (grown for 18 hr) showed that GlnRS and TyrRS are overproduced 30- and 10-fold, respectively. Thus, a 10-fold overproduction of TyrRS is enough to abolish mischarging of *supF* $tRNA^{Tyr}$ caused by a 30-fold elevated GlnRS level.

Aminoacylation

Since the *in vivo* misacylation assay is based on functional nonsense suppression which involves all the steps of protein biosynthesis, the *in vivo* competition experiments do not allow us to attribute the decrease in mischarging directly to the effect competition has on the accuracy of aminoacylation. However, the fact that the increase in accuracy of translation occurs when overexpression of either the cognate tRNA ($tRNA^{Gln}$) for the mischarging enzyme (2) or the cognate enzyme (TyrRS) for the mischarged tRNA (*supF* $tRNA^{Tyr}$) is concurrent with the overproduction of GlnRS (Fig. 2) argues that it is the accuracy of the aminoacylation event itself that is enhanced. In order to show specifically that the accuracy of aminoacylation is dependent

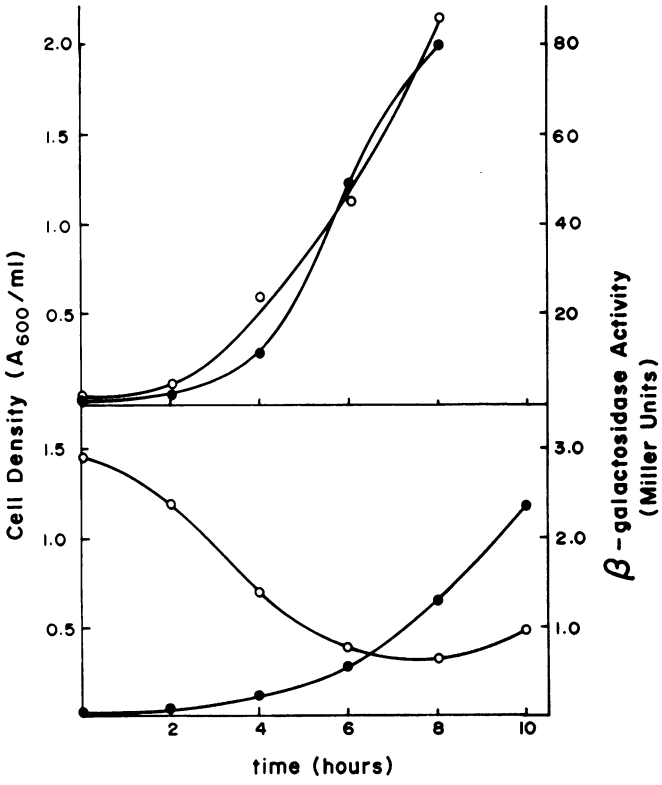

Figure 2. Overproduction of TyrRS abolishes *supF* Gln-tRNA^{Tyr} formation *in vivo*. Cell density (●,A_{600}) and β-galactosidase activity (○, Miller units) were measured for four independent isolates of strain BT32 in which either GlnRS alone is overexpressed on pRS11 and pBR322 is present as a control (top panel) or GlnRS and TyrRS (pBRTyrTS) are concurrently overexpressed (bottom panel).

on the relative concentrations, and thus the degree of complexation of tRNA and its cognate synthetase, we tested the competition using *in vitro* aminoacylation reactions with purified enzymes and tRNAs. To determine the acceptor activities of the various tRNAs for glutamine as well as for their cognate amino acids, aminoacylation of the tRNAs by GlnRS as well as by their cognate synthetases was determined.

Earlier work demonstrated that both *supF* tRNA^{Tyr} (32) and tRNA^{Glu} (37) could be mischarged by GlnRS *in vitro* and thus may be considered 'dual identity' tRNAs. In order to examine this in more detail we determined the glutaminylation and/or tyrosylation of tRNA^{Glu}, wild-type tRNA^{Tyr}, the normal *supF* tRNA^{Tyr} (with A in position 73), and the mutant, *supF* tRNA^{Tyr}G73 (Fig. 1). As Table I shows, the *supF* tRNA^{Tyr} (anticodon CUA) is charged more poorly than wild-type tRNA^{Tyr} (anticodon QUA) and that a change in the discriminator base (position 73) of the suppressor tRNA abolishes charging with tyrosine. The reduced affinity of TyrRS for *supF* tRNA^{Tyr} as compared with wild-type tRNA^{Tyr} is in agreement with previously published results (22). In agreement with other results (15,38,39), it appears that the discriminator base and the anticodon are involved in the tRNA recognition by TyrRS. In order to determine the extent of loss of tyrosine identity caused by these changes it will be necessary to perform detailed enzyme-kinetic analyses of the substrate properties of the pure tRNA species (obtained by *in vitro* T7 RNA polymerase transcription). The mischarging with glutamine of the three tRNA^{Tyr} acceptors and tRNA^{Glu} shows that while tRNA^{Glu} can be mischarged it is

Table I. Acylation and misacylation of *E. coli* tRNAs[a)]

tRNA	Enzymes GlnRS	TyrRS	GluRS
wild-type tRNA^{Tyr}	124	1473	nd
*supF*tRNA^{Tyr}A73	710	679	nd
*supF*tRNA^{Tyr}G73	1310	151[b]	nd
tRNA^{Glu}	612	nd	1450

a) Acceptor activities of tRNA (pmol/A_{260}).
b) This value is presumed to be due to wild-type tRNA^{Tyr} contamination (Figure 2).
nd=not determined.
Aminoacylation was carried out as described in Materials and Methods. The reactions were incubated for 31 min at 37°C at which time a plateau was reached. For glutamine charging, the tRNA concentration was 1.0 μM and a 2-fold excess of GlnRS was used. [^{14}C]glutamine was present at 150 μM with specific activities from 76–99 cpm/pmol. For tyrosine charging, tRNA concentrations were 0.08 μM and 0.16 μM for wild-type tRNA^{Tyr} and the *supF* tRNA^{Tyr} species respectively, and, in all cases the tRNAs were present at a 50-fold molar excess over TyrRS. [^{14}C]tyrosine was present at 60 μM with specific activities ranging from 282–733 cpm/pmol.

not as good a substrate for GlnRS as the derivatives of *supF*. This is probably explained by the fact that *supF* tRNA^{Tyr}G73, which is misacylated to 90%, shares more of the major acceptor stem and anticodon identity elements of tRNA^{Gln} which make direct contacts with GlnRS (11,12,14) than does tRNA^{Glu}. It is also pertinent to note that the dual identity of the mutant tRNA^{Tyr} species is evident and that each change (Q34→C in the anticodon and A73→G in the discriminator) successively reduces the tyrosine identity and concomitantly strengthens glutamine identity so that the *supF* tRNA^{Tyr}G73 is mainly acylated with glutamine (Table I).

In vitro competition assays

We wanted to test whether the effects of the competition by synthetases for tRNA observed *in vivo* could be duplicated *in vitro*. Thus, two *in vitro* competition experiments were carried out; one in which GluRS was used to prevent Gln-tRNA^{Glu} formation, and the other employing TyrRS to block Gln-tRNA^{Tyr} formation. For this purpose, aminoacylation of tRNA^{Glu} and the tRNA^{Tyr} species by GlnRS with glutamine was examined in the presence and absence of the cognate aminoacyl-tRNA synthetase (Fig. 3 and 4).

GluRS competes extremely well with GlnRS for tRNA^{Glu} and could abolish almost all observable mischarging so long as sufficient quantities of GluRS were added (Fig. 3). All observable mischarging was abolished when 70 nM GluRS competed with 2 μM GlnRS for 5 μM tRNA^{Glu} (Fig. 3A). Most likely, the small amount of residual charging observed under these conditions is due to contaminating tRNA^{Gln} in the commercial tRNA^{Glu} preparation.

Is the increase in accuracy of aminoacylation due to competition a specific effect, *i.e.*, must the competing synthetase be the cognate one for the mischarged tRNA? We chose MetRS, which does not charge tRNA^{Glu} (data not shown), as the non-specific competitor. As can be seen in Fig. 3, mischarging of tRNA^{Glu} was unaltered in the presence of MetRS. Clearly, a competing synthetase must have significant affinity for the particular tRNA species.

In our experiments with TyrRS, we found that TyrRS competes effectively with GlnRS for *supF* tRNA^{Tyr}A73 (Fig. 3) under the conditions employed. However, this applies only to the real 'dual

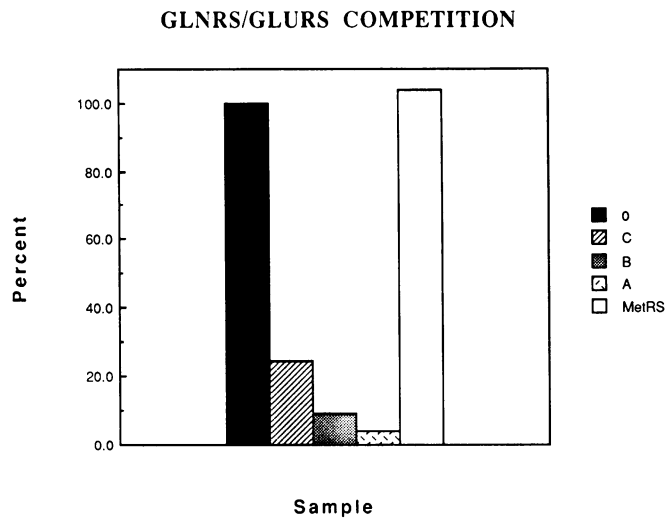

Figure 3. Addition of GluRS diminishes *in vitro* mischarging of $tRNA^{Glu}$ by GlnRS. The concentration of $tRNA^{Glu}$ was 5.0 μM, while the concentration of GlnRS was 2.0 μM. The specific activity of the [^{14}C]glutamine was 22 cpm/pmol. GluRS, and MetRS when present, was added simultaneously with GlnRS in the following amounts: 0) 0 pmol, A) 4.23 pmol, B) 0.85 pmol, C) 0.17 pmol and MetRS) 0.67 pmol.

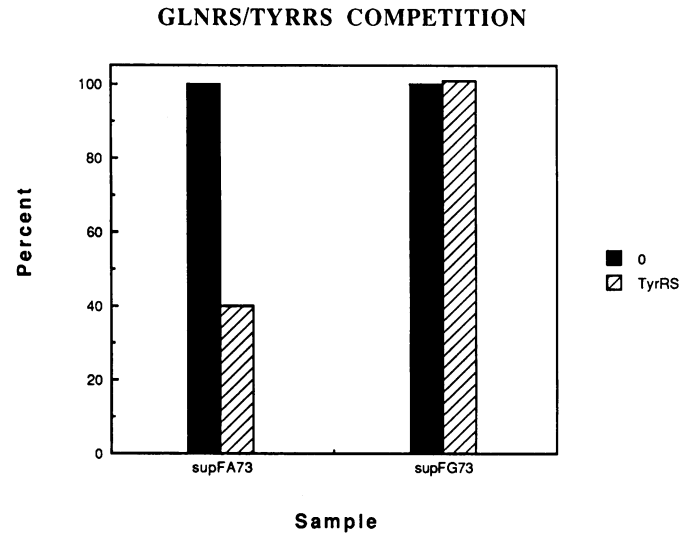

Figure 4. Effect of TyrRS addition on *in vitro* mischarging by GlnRS of $tRNA^{Tyr}$ species. Both of the *supF* $tRNA^{Tyr}$ species were present at 1.0 μM concentrations, which was a two-fold molar excess of tRNA over GlnRS. In all cases, when TyrRS [0) 0 pmol, TyrRS) 17.0 pmol] was present, it was added simultaneously with the GlnRS and MetRS. The measured specific activities of the [^{14}C]glutamine varied slightly between experiments (77–92 cpm/pmol).

identity' *supF* $tRNA^{Tyr}A73$ (Table I); the glutaminylation of *supF* $tRNA^{Tyr}G73$ could not be suppressed by TyrRS under the experimental conditions used. This was expected from the apparent inability of TyrRS to recognize and aminoacylate *supF* $tRNA^{Tyr}G73$ *in vitro* (Table I). In these experiments, MetRS was added concurrently with GlnRS to all the assays in order to rule out the possibility of non-specific competition. The same decrease (60%) in mischarging of *supF* $tRNA^{Tyr}$ by GlnRS upon addition of TyRS could be seen in the assays done in the absence of MetRS (data not shown).

It is interesting to note the relative ability of TyrRS and GluRS to compete with GlnRS for their respective substrates. While a 75% decrease in glutaminylation of $tRNA^{Glu}$ is observed in the presence of >720-fold molar excess of GlnRS to GluRS, a 60% decrease is observed with a 2-fold molar excess of GlnRS to TyrRS. Presumably, GluRS competes more effectively than TyrRS because GluRS has a higher affinity for $tRNA^{Glu}$ than TyrRS has for *supF* $tRNA^{Tyr}$.

DISCUSSION

The fact that elevated levels of TyrRS *in vivo* can abolish the mischarging of *supF* $tRNA^{Tyr}A73$ by overproduced GlnRS have confirmed that the balance of the amounts of aminoacyl-tRNA synthetase and tRNA is critical for faithful protein translation. Not only can the overproduction mischarging phenomenon be abolished by complexation of the GlnRS by overexpressing its cognate substrate $tRNA^{Gln}$, it can also be abolished by sequestering the mischarged *supF* $tRNA^{Tyr}$ by overexpressing its cognate synthetase, TyrRS. Thus, intracellularly, it is the amount of free (uncomplexed) substrate, *supF* $tRNA^{Tyr}$, as well as free enzyme which determines the accuracy with which aminoacyl-tRNA formation occurs.

The *in vitro* competition experiments demonstrate directly that the competition occurs at the aminoacylation step and that both TyrRS and GluRS can decrease the levels of mischarging by GlnRS of *supF* $tRNA^{Tyr}$ and $tRNA^{Glu}$ respectively. However, it appears that the relative affinities of the synthetases, as well as their relative levels, may play a role in the accuracy of aminoacyl-tRNA formation. GluRS has a greater affinity for $tRNA^{Glu}$ and thus can compete more effectively against GlnRS for $tRNA^{Glu}$ than TyrRS can for *supF* $tRNA^{Tyr}$. Likewise, TyrRS cannot compete with GlnRS for the discriminator mutant of *supF* $tRNA^{Tyr}$ (*supF* $tRNA^{Tyr}G73$), for which it has little or no apparent affinity. However, it is expected that TyrRS would be as effective a competitor for wild-type $tRNA^{Tyr}$ as GluRS is for $tRNA^{Glu}$. The competition effect is a specific one in that an unrelated synthetase, MetRS, cannot compete with GlnRS for any of the mischarged tRNAs tested. Although we have not directly tested whether an editing activity of GluRS and/or TyrRS accounted for the observed decrease in the mischarging of their respective cognate tRNAs by GlnRS, we consider this unlikely as neither of the synthetases in question has been demonstrated to have a proofreading activity (reviewed in 40). In fact, it has been shown that TyrRS compensates for its lack of editing activity by employing very specific mechanisms for recognition and activation of tyrosine (41) and GluRS, like GlnRS, has a very high K_M for its cognate amino acid (37,42,43).

Unlike the standard *in vivo* methods used to study tRNA identity, *in vitro* competition can be applied to non-suppressor tRNAs (thus eliminating any bias toward recognition of the suppressor anticodons by a specific subset of synthetases) and allows one to examine the aminoacylation event exclusively. The *in vitro* competition technique does not require additional purified enzymes or substrates beyond those needed for current *in vitro* methods. Thus, it could be used to examine the effect of mutations, even anticodon mutations, on tRNA identity, *e.g.*, the identity of $tRNA^{Tyr}$-G73 (anticodon QUA) could be compared with that of *supF* $tRNA^{Tyr}G73$ (anticodon CUA). In addition, those anticodon mutants which do not confer methionine or other 'initiation-competent' amino acid identity and thus are

not assayable in the initiation missense suppression assays (25,27) could be analyzed in this way. Finally, mutants of aminoacyl synthetases could also be examined in the *in vitro* competition system.

The aminoacylation results for the three $tRNA^{Tyr}$ species demonstrate that the discriminator base and the first base of the anticodon are important for recognition by *E. coli* tyrosyl-tRNA synthetase (Table I). This was already noted for the discriminator position (15,38,39), while the second position of the anticodon has also been shown to be important for tyrosine charging (15).

ACKNOWLEDGEMENTS

We thank Kelley Rogers and Sergey Beresten for helpful scientific discussions. This work was supported by a grant from NIH.

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