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# Selection of suppressor methionyl-tRNA synthetases: Mapping the tRNA anticodon binding site

(aminoacyl-tRNA synthetase/protein–nucleic acid interactions/tRNA discrimination/site-directed mutagenesis)

THIERRY MEINNEL, YVES MECHULAM*, DANIEL LE CORRE, MICHEL PANVERT, SYLVAIN BLANQUET, AND GUY FAYAT†

Laboratoire de Biochimie, Unité Associée 240 du Centre National de la Recherche Scientifique, Ecole Polytechnique, 91128 Palaiseau Cedex, France

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ABSTRACT **Accurate aminoacylation of a tRNA by *Escherichia coli* methionyl-tRNA synthetase (MTS) is specified by the CAU anticodon. A genetic screening procedure was designed to isolate MTS mutants able to aminoacylate a methionine amber tRNA (CUA anticodon). Selected suppressor MTS enzymes all possess one or several mutations in the vicinity of Trp-461, a residue that is the major contributor to the stability of complexes formed with tRNAs having the cognate CAU anticodon. Analysis of catalytic properties of purified suppressor enzymes shows that they have acquired an additional specificity toward the amber anticodon without complete disruption of the methionine anticodon site. It is concluded that both positive and negative discrimination toward the binding of tRNA anticodon sequences is restricted to a limited region of the synthetase, residues 451–467.**

Recognition of a tRNA by its cognate aminoacyl-tRNA synthetase depends on a restricted number of nucleotide determinants (reviewed in ref. 1). Aminoacyl-tRNA synthetase/tRNA systems can be classified according to the contribution of the tRNA anticodon to the discrimination process.

(*i*) In two cases, tRNA identity elements were clearly localized outside the anticodon: at a single base pair, G3·U70, in the case of the *Escherichia coli* alanine system (2–6), and at eight positions on $tRNA^{Ser}$ (1, 7).

(*ii*) Mixed situations are encountered, where both the anticodon sequence and other positions on the tRNA direct aminoacylation by the synthetase (e.g., $tRNA^{Phe}$ from yeast; ref. 8). Two *E. coli* aminoacyl-tRNA synthetases (Gln and Lys) belonging to this second class exhibit the peculiar ability to misacylate various amber suppressor tRNAs (1). Resolution of the three-dimensional structure of the glutaminyl-tRNA synthetase/tRNA complex confirmed the role of the central base of the anticodon in tRNA discrimination and ascertained the importance of additional interactions at the level of the acceptor arm (9). *In vivo* selection of mischarging glutaminyl-tRNA synthetases exhibiting a broader specificity substantiated the idea that these acceptor-arm contacts are critical in directing tRNA discrimination (10, 11).

(*iii*) The *E. coli* methionine system represents a third class, in which the anticodon itself appears sufficient to direct recognition by the synthetase, since methionine-accepting ability is lost upon anticodon modification of $tRNA_f^{Met}$ (12) and can be conferred on non-Met tRNA species provided that they have been given a CAU anticodon (13, 14). This conversion of tRNA identity was evidenced *in vitro* by changing the UAC anticodon of $tRNA_1^{Val}$ to CAU. In addition, the precursor of $tRNA^{Ile}_{minor}$, which naturally carries a CAU anticodon, behaves as a methionine acceptor *in vitro*.

How methionyl-tRNA synthetase (MTS) recognizes the CAU anticodon determinant remains unsolved. However, the region of Lys-465 has been implicated in anticodon recognition (15–17). Here we show that a single amino acid replacement (Asp-456 → Tyr) is sufficient to permit aminoacylation of a methionine amber tRNA *in vivo*. In addition, Trp-461 of *E. coli* MTS is shown to be the major contributor to the *in vitro* stability of complexes with tRNAs possessing a CAU anticodon as their only common feature. We conclude that a limited peptide region of MTS comprising the crucial aromatic residue Trp-461 accounts for both positive and negative discrimination among the various tRNA anticodon sequences.

## MATERIALS AND METHODS

**Construction and Purification of MTS Variants.** Oligonucleotide-directed mutagenesis (18) was used to introduce mutations into the *metG547* gene, which encodes the monomeric variant of MTS (M547, 547 residues), cloned into pBSM13-KS (19). DNA sequence of each variant gene was completely determined using a set of 12 synthetic oligonucleotidic primers. M547 variants were purified as described (19) except that Mono Q (Pharmacia) was used as the ion exchanger. The mutant M547 proteins were named by indicating the amino acid transition (one-letter symbols) and its position [e.g., M547WA461 (or simply WA461) has Trp-461 replaced by alanine].

**Construction of tRNA Genes.** The genes were assembled from six overlapping oligonucleotides and cloned into the vector pBSTNAV (20). The $tRNA^{Ile}_{(CUA)}$ gene was also cloned into plasmid pACYC184, yielding pACileCUA. λmetCUA and λvalCUA are derivatives of λMH20 (*imm21*) (21) carrying the gene for $tRNA^{Met}_{m(CUA)}$ or $tRNA^{Val}_{1(CUA)}$ under the control of the *lpp* promoter.

**tRNA Purification.** Each overproduced tRNA [Ile GAU, Met elongator (m), Met initiator (f), Met elongator CUA, Met initiator CUA, and Ile CAU] was purified by liquid chromatography (20). In the case of $tRNA^{Ile}_{(CAU)}$, $tRNA^{Met}_{m(CUA)}$, and $tRNA^{Met}_{f(CUA)}$, great care was taken to eliminate the wild-type methionine- and isoleucine-accepting tRNA species. Concentrations of $tRNA^{Ile}_{(CAU)}$ and $tRNA^{Met}_{(CUA)}$ (f or m) were routinely determined by dot blot hybridization with specific oligonucleotide probes. The concentrations were deduced from radioactivity measurements and comparison with purified non-mutant tRNA samples of known concentrations. Concentrations (pmol/$A_{260}$ unit) were 1000 ± 200 for $tRNA^{Ile}_{(CAU)}$ and 1050 ± 100 for $tRNA^{Met}_{(CUA)}$; contaminations in these preparations were 1 ± 0.2 for $tRNA_m^{Met}$ and 5 ± 0.5 for $tRNA_f^{Met}$ in the case of $tRNA^{Ile}_{(CAU)}$ and 10 ± 5 for $tRNA^{Met}$ in the $tRNA^{Met}_{(CUA)}$ species. Methionine-accepting activities,

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Abbreviation: MTS, methionyl-tRNA synthetase.

*To whom reprint requests should be addressed.
†Deceased, October 22, 1990.

measured in the standard aminoacylation assay (see below) in the presence of 1.5 μM M547 enzyme, were 1280 ($tRNA_m^{Met}$), 1350 ($tRNA_f^{Met}$), 850 [$tRNA_{(CAU)}^{Ile}$] and 12 [$tRNA_{major(GAU)}^{Ile}$] pmol/$A_{260}$ unit. Isoleucine acceptance by $tRNA_{major(GAU)}^{Ile}$, measured in the presence of 1.2 μM isoleucyl-tRNA synthetase, was 1040 pmol/$A_{260}$ unit.

**Enzyme Assays.** Dissociation constants ($K_d$) of the enzyme/tRNA complexes were determined spectrophotofluorimetrically by titration of an enzyme solution (0.8 μM) with increasing tRNA concentrations (19, 22). Unless otherwise stated, $k_{cat}$ ($V_{max}/[E_0]$) and $K_m$ values were obtained from aminoacylation assays at 25°C in 100 μl of 20 mM Tris·HCl, pH 7.6/7 mM $MgCl_2$/10 mM 2-mercaptoethanol/0.1 mM EDTA/2 mM ATP/150 mM KCl with 21.5 μM [*methyl*-$^{14}$C]methionine (50.3 Ci/mol; 1 Ci = 37 GBq) or [*methyl*-$^{3}$H]methionine (1000 Ci/mol) (Commissariat à l'Energie Atomique, Saclay, France) and various concentrations of the tRNA under study (0.02–25 μM) (19, 23).

**Selection of Suppressor Variants of M547.** The indicator strain 121R [*ara*, *argE*am, Δ(*lac–proB*), *nalA*, *rpoB*, *thi*, *recA56*,*srl300*:Tn*10*, F′(*proA*$^+$*B*$^+$, *lacI–Z*am*181*] (24, 25) was lysogenized with a single copy of λmetCUA. To prepare a randomly mutagenized library, the DNAs (1 μg) of pBSM547 plasmid derivatives expressing the M547 variants were used to transform $10^9$ cells of the mutator strain JM101MutD (gift of M. Springer, IBPC, Paris) by electroporation. From $10^7$ to $10^8$ transformants were used to inoculate 5 ml of LB medium containing ampicillin (50 μg/ml). After overnight growth at 37°C (8–10 doublings), plasmid DNA was extracted and used to transform the indicator strain (121R-λmetCUA). Cells were plated onto selective M9 plates containing ampicillin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (40 μg/ml). Transformation efficiency was evaluated in parallel by plating dilutions on the same medium supplemented with arginine. Arg$^+$ blue colonies were scored after 48–60 hr of incubation. To verify that the phenotype was linked to the enzyme expression, plasmid DNA was prepared from the positive clones and used to transform the indicator strain. The positive plasmids were purified and the complete sequences of the *metG* variant genes were determined.

**Quantitative Determination of Suppression Efficiency.** The indicator strains expressing suppressor M547 variants were grown in M9 minimal medium supplemented with ampicillin (50 μg/ml); in the case of Arg$^-$ strains, arginine was added (24 μg/ml). Two aliquots (1.6 ml) of each culture were withdrawn during exponential growth (0.5 and 0.9 $OD_{650}$ unit). β-Galactosidase (26) and MTS (19) were assayed as described. The β-galactosidase activity measured in the absence of MTS overproduction (0.6 unit, strain UF121R-λmetCUA) was subtracted from the measured β-galactosidase specific activities, and suppression efficiency was obtained after normalization to the intracellular MTS concentration as evaluated from the methionine-dependent ATP/$PP_i$ exchange activity measured in the extracts.

## RESULTS

**Anticodon of tRNA Directs MTS/tRNA Complex Formation.** The GAU isoleucine anticodon of $tRNA_{major(GAU)}^{Ile}$ was converted to the CAU methionine anticodon, giving $tRNA_{(CAU)}^{Ile}$. $tRNA_{major(GAU)}^{Ile}$ was not recognized by MTS (Table 1). However, a single G → C transversion changing the GAU anticodon to CAU converted the tRNA into a good substrate for the fully active monomeric MTS composed of 547 residues (M547), while abolishing its aminoacylation by isoleucyl-tRNA synthetase ($k_{cat}/K_m < 1\ M^{-1}s^{-1}$; Table 1).

This identity swap appeared essentially driven by the formation of a stable enzyme/tRNA complex, since the $K_d$ of the M547/$tRNA_{(CAU)}^{Ile}$ complex was even smaller than that of the M547/$tRNA_m^{Met}$ complex (Table 1). Reciprocally, the change of the CAU anticodon of $tRNA_m^{Met}$ to the amber anticodon (CUA) precluded its recognition by MTS (Table 1). These results and previous ones (12–14) clearly establish that the CAU anticodon is a determinant for the formation of a stable MTS/tRNA complex. This behavior permits the design of a genetic screening procedure aimed at selecting MTS mutants with a modified tRNA anticodon specificity.

Table 1. Parameters of the tRNA aminoacylation reaction catalyzed by M547 MTS

| Substrate | $K_m$, μM | $K_d$, μM | $k_{cat}$, $s^{-1}$ | $k_{cat}/K_m$, $M^{-1}s^{-1}$ |
|---|---|---|---|---|
| $tRNA_{m\ or\ f}^{Met}$ | 0.02 ± 0.01 | 0.43 ± 0.10 | 0.33 ± 0.05 | $16.5 \times 10^6$ |
| $tRNA_{(CAU)}^{Ile}$ | 0.05 ± 0.02 | 0.16 ± 0.04 | 0.12 ± 0.01 | $2.4 \times 10^6$ |
| $tRNA_{m(CUA)}^{Met}$ | >100 | >15 | NM | <1 |
| $tRNA_{(GAU)}^{Ile}$ | >100 | >15 | NM | <1 |

$k_{cat}$ ($V_{max}/[E_0]$) and $K_m$ values were obtained from standard aminoacylation assays at 25°C in the absence of added KCl, with M547 as the catalyst and the indicated tRNA as the substrate. $K_d$ values for the enzyme/tRNA complexes were determined spectrofluorimetrically. Values were identical for both $tRNA_f^{Met}$ and $tRNA_m^{Met}$. Aminoacylation efficiency ($k_{cat}/K_m$) is the slope at the origin of the $V_i/[E_0]$ vs. [tRNA] plot ($[E_0]$ is the total enzyme concentration). Lowest detectable aminoacylation efficiency was 1 $M^{-1}s^{-1}$. NM, not measurable.

**A Single Substitution at Position 456 Makes MTS a "Suppressor" Enzyme *in Vivo*.** The screening procedure is based on the suppression of amber mutations interrupting the translation of two indicator genes, *argE* and a *lacI–lacZ* fusion. Insertion of a single copy of the $tRNA_{m(CUA)}^{Met}$ gene into the strain 121R was chosen because the background suppression by the other aminoacyl-tRNA synthetases known to misacylate amber suppressor tRNAs (1, 27) was proportional to the tRNA gene-copy number (data not shown). In this genetic context, the lysogenic strain remained an arginine auxotroph and expressed a very low level of β-galactosidase activity (0.6 Miller unit). Overproduction of wild-type M547 from the pBSM547 plasmid did not relieve the arginine auxotrophy and only slightly increased β-galactosidase activity (2.8 Miller units). This establishes that wild-type MTS does not efficiently aminoacylate the amber tRNA *in vivo*.

A randomly mutagenized progeny of the pBSM547 plasmid was used to transform 121R-λmetCUA (Fig. 1). Of $10^9$ transformants, 50 had become arginine prototrophs and formed blue colonies. However, among the 50 plasmids prepared from these clones, only 1 could relieve the arginine auxotrophy. Sequencing of the corresponding *metG* gene revealed a single transversion, resulting in the replacement of Asp-456 by tyrosine.

That methionylation of the amber $tRNA_m^{Met}$ can be achieved through a single mutation at position 456 may reflect a direct involvement of this region in anticodon recognition. Indeed, previous work from this laboratory (16) and others (17) indicates that Trp-461 might be involved in anticodon recognition. Upon replacement of Trp-461 by phenylalanine, the $K_m$ of $tRNA^{Met}$ increased by a factor of at least 500. This effect was mainly caused by a reduction of tRNA affinity (Table 2). Another mutant enzyme was constructed by replacing Trp-461 with alanine (M547WA461). This substitution by a nonaromatic residue caused a further 100-fold decrease in catalytic efficiency ($k_{cat}/K_m$) and rendered the $K_m$ and $K_d$ of tRNA, initiator or elongator, immeasurably high (Table 2).

These results show that Trp-461 is required for complex formation, through a direct interaction with the CAU anticodon sequence and/or the stabilization of the proper three-dimensional structure in the Trp-461 region. Accordingly, the loss of Trp-461 should affect the binding of other tRNAs carrying the CAU anticodon to the same extent, regardless of

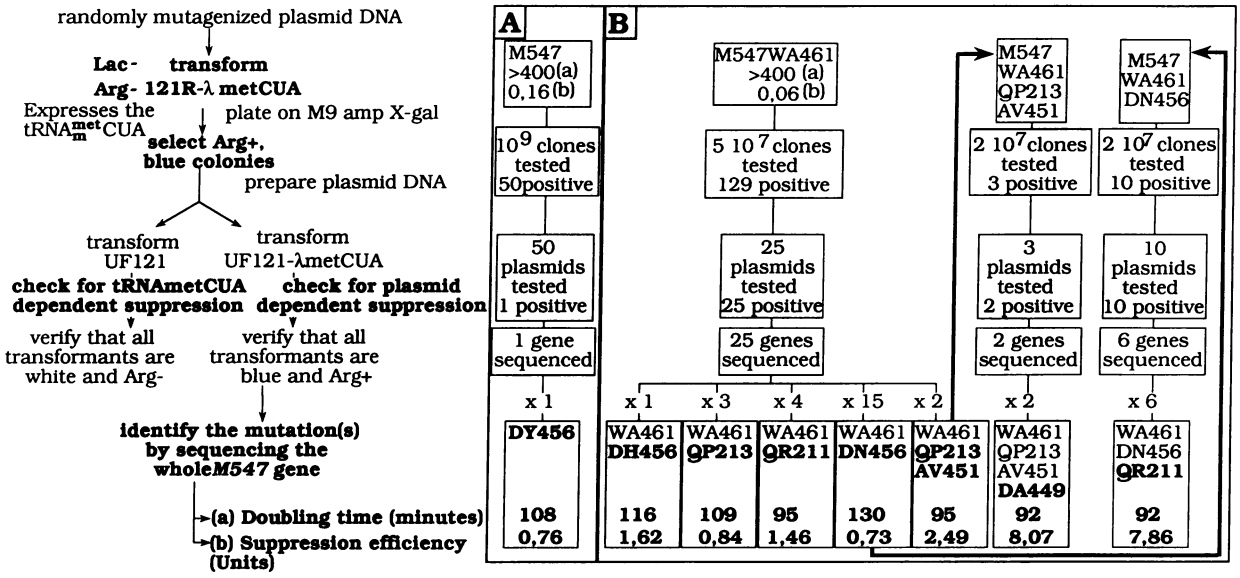


FIG. 1. Genetic screening procedure for the selection of suppressor MTS. The procedure is summarized at left. amp, Ampicillin; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside. Four starting enzymes (top of *A* and *B*) were used: two nonsuppressors (M547 and M547WA461) and two suppressors (M547WA461QP213AV451 and M547WA461DN456). The number of clones selected after each step is indicated. Indicated at the bottom are the obtained mutation (boldface) and the doubling time and β-galactosidase activity of the host strain (see *Materials and Methods*). Corresponding values for the M547 and M547WA461 starting enzymes are indicated below the enzyme names: doubling time (a) and β-galactosidase activity (b).

the rest of their primary structures. Indeed, the efficiencies of aminoacylation of $tRNA^{Ile}_{(CAU)}$ by the WF461 and WA461 mutant enzymes were reduced by roughly the same factors as those measured in the case of the aminoacylation of $tRNA^{Met}$ (Table 2). Again, the lowering of the catalytic efficiency of WF461 in the methionylation of $tRNA^{Ile}_{(CAU)}$ reflects mainly a loss of tRNA affinity (Table 2).

**Trp-461 Acts as a Negative Discriminator Against the Amber Anticodon.** The low frequency of appearance ($10^{-9}$) of one suppressor MTS might mean that conservation of the essential Trp-461 residue restricts the number of mutations capable of yielding suppressor enzymes. To examine this possibility, the genetic screening procedure for isolating suppressor MTS was carried out starting from the gene expressing the mutant M547WA461 enzyme. In this case, a 2500-fold increase in the frequency of suppressor plasmid occurrence was observed (Fig. 1*B*).

Among the 25 selected mutant plasmids, 16 carried a single additional mutation at position 456. These included 15 G → A transitions (Asp → Asn) and one G → C transversion (Asp → His; Fig. 1*B*), the latter being more efficient in terms of suppression than the former. A second locus (211 region) emerged from the analysis of the 9 remaining clones: a Gln-211 → Arg replacement was found three times and a Gln-213 → Pro change four times. Finally, a clone combining mutations in both regions (Gln-213 → Pro and Ala-451 → Val) was found twice. This variant, WA461QP213AV451, exhibited a higher suppression efficiency than the WA461QP213 mutant.

One of the suppressors obtained, M547WA461DN456, was mutagenized and submitted to a new cycle of selection. Much better suppressors were found at a frequency of $5 \times 10^{-7}$, and, of the six genes analyzed, all carried a mutation in the 211 region (Gln-211 → Arg; Fig. 1*B*). Submission of WA461QP213AV451 to a further selection (Fig. 1) yielded at high frequency a more efficient suppressor that carried one more mutation, in the 460 region (Asp-449 → Ala). Finally, *in vitro* construction of the WA461DH456QR211 mutant resulted in a suppressor enzyme even better than the WA461DN456QR211 variant, consistent with the higher gain of suppression efficiency brought by the histidine substitution at position 456 (Fig. 2).

Two representative suppressor MTS enzymes, DY456 and WA461DH456QR211, were purified to determine their catalytic properties in the aminoacylation of the amber (CUA) derivative of $tRNA^{Met}_m$. The efficiency ($k_{cat}/K_m$) of $tRNA^{Met}_{m(CUA)}$ aminoacylation by WA461DH456QR211 was 9-fold higher than that by DY456 (Fig. 3, black bars). This factor paralleled the corresponding 13-fold increase of suppression efficiency measured *in vivo* for the two enzymes (Fig. 2). Methionylation efficiencies were also determined for tRNA substrates having a methionine anticodon [$tRNA^{Met}$ (m and f) and $tRNA^{Ile}_{(CAU)}$; Fig. 3]. The gain in amber tRNA methionylation efficiency due to the single mutation DY456 was accompanied by a decrease in $k_{cat}/K_m$ for the two wild-type methionine tRNAs and $tRNA^{Ile}_{(CAU)}$.

Table 2. Mutations at position 461 affect methionine anticodon recognition

| Enzyme | Substrate | $k_{cat}/K_m$ $M^{-1}s^{-1}$ | Relative $k_{cat}/K_m$ | $K_d$, μM | Relative affinity |
|---|---|---|---|---|---|
| M547 | $tRNA^{Met}_{m\ or\ f}$ | $16.5 \times 10^6$ | 1 | 0.43 | 1 |
| | $tRNA^{Ile}_{(CAU)}$ | $2.4 \times 10^6$ | 1 | 0.16 | 1 |
| WF461 | $tRNA^{Met}_{m\ or\ f}$ | 50,000 | $3.3 \times 10^{-3}$ | 25 ($K_m$) | $17 \times 10^{-3}$ |
| | $tRNA^{Ile}_{(CAU)}$ | 2,000 | $0.8 \times 10^{-3}$ | 100 ($K_i$) | $4 \times 10^{-3}$ |
| WA461 | $tRNA^{Met}_{m\ or\ f}$ | 400 | $2.7 \times 10^{-5}$ | NM | NM |
| | $tRNA^{Ile}_{(CAU)}$ | 26 | $1.1 \times 10^{-5}$ | NM | NM |

$k_{cat}/K_m$ values were obtained from standard aminoacylation assays at 25°C in the absence of added KCl, with M547, M547WF461, and M547WA461 as catalysts and the indicated tRNAs as substrates. Lowest measurable $k_{cat}/K_m$ value was 1 $M^{-1}s^{-1}$. $K_d$ values for the enzyme/tRNA complexes were determined spectrofluorimetrically. With the WF461 enzyme, the fluorescence experiments indicated $tRNA_f^{Met}$ and $tRNA_m^{Met}$ $K_d$ values >15 μM. In these cases, measured $K_m$ values may be regarded as good approximations of the $K_d$ values. The equilibrium constant of $tRNA^{Ile}_{(CAU)}$ for the WF461 enzyme was estimated from the inhibition constant ($K_i$) of $tRNA^{Ile}_{(CAU)}$ in the aminoacylation of $tRNA^{Met}_m$. NM, not measurable.

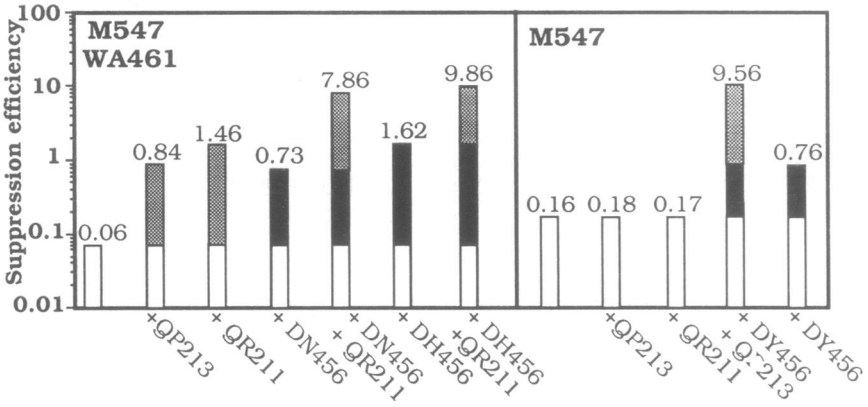


FIG. 2. Role of mutations in the 460 region and 211 region. Below each vertical bar is indicated the additional mutation(s) to either the M547WA461 species (*Left*) or the M547 species (*Right*). For each variant, the suppression efficiency measured *in vivo* is indicated above the vertical bar. The gain in suppression efficiency brought by each added mutation is shown: stippled bar, mutation at 211 or 213; black bar, mutation at 456. Open bar represents the suppression efficiency of the starting enzyme (M547WA461 or M547).

**At Least One Mutation in the 460 Region Is Required for Creating the Site of an Amber Anticodon.** No region-211 mutant was selected from wild-type M547, suggesting that, as opposed to the DY456 mutation, mutation in the 211 region could not confer the suppressor phenotype. This was confirmed by determining the phenotype resulting from overproduction of either QR211 or QP213 after construction of the corresponding genes *in vitro*. These two mutants, which still have Trp-461, did not relieve the $Arg^-$ phenotype. Their suppression efficiencies remained equal to that of the wild-type enzyme (Fig. 2). Consistent with this, these two nonsuppressor enzymes remained unable to methionylate the amber tRNAs *in vitro* (Fig. 3). We conclude that mutations in the 211 region are not able to confer on MTS the ability to recognize an amber anticodon. This means that mutations in the 211 region may stimulate the efficiency of suppression—provided that a change in the 460 region is present—by, for instance, increasing the rate of tRNA aminoacylation regardless of the sequence of the anticodon. To probe this hypothesis, pure QR211 and QP213 enzymes were produced and their methionylation efficiencies ($k_{cat}/K_m$) were determined using three tRNA substrates possessing the methionine anticodon [$tRNA^{Met}$ (m and f) and $tRNA^{Ile}_{(CAU)}$]. While their affinity for $tRNA^{Met}_m$ was not changed (QR211, 0.3 ± 0.1 μM; QP213, 0.2 ± 0.1 μM, as compared to M547, 0.4 ± 0.1 μM), the two region-211 mutants catalyzed the methionylation of these three tRNAs at least 10-fold more efficiently than did the M547 enzyme (Fig. 3). Therefore, these mutations are likely to accelerate the rate of aminoacylation independently of the anticodon sequence.

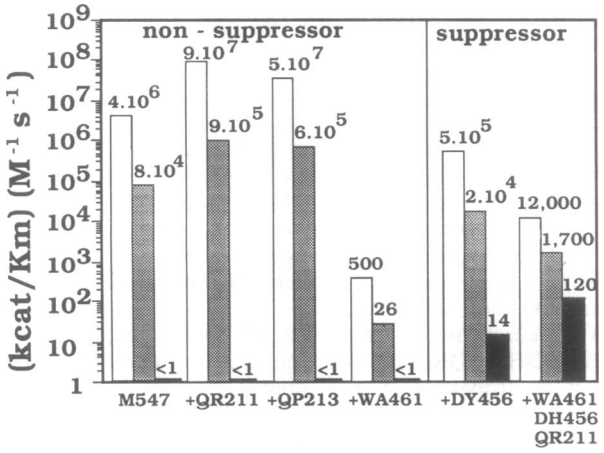


FIG. 3. Specificity of the suppressor enzymes. Represented are the *in vitro* catalytic efficiencies toward the indicated tRNA substrates, measured in the presence of 150 mM KCl: open bars, $tRNA^{Met}$ (f or m; values were identical for both); stippled bars, $tRNA^{Ile}_{(CAU)}$; black bars, amber $tRNA^{Met}$ (f or m; values were identical for both). The enzymes were M547 derivatives containing the indicated mutation(s). Aminoacylation efficiency ($k_{cat}/K_m$), indicated above each bar, is the slope at the origin of the $V_i/[E_0]$ vs. [tRNA] plot ($[E_0]$ is the total enzyme concentration).

As expected from the above results, subsequent introduction of the DY456 mutation into the M547QP213 mutant gene yielded a suppressor enzyme variant (M547DY456QP213) whose suppression efficiency was 12-fold that observed with the DY456 mutation alone (Fig. 2, black bars). The magnitude of this gain (stippled bars) is of the order of that (14-fold) obtained upon introduction of the QP213 mutation in M547WA461. In fact, it is similar to the factor of acceleration of the tRNA methionylation rate measured *in vitro* (Fig. 3).

We therefore conclude that mutations in only the 460 region (i.e., either at Trp-461 or a second-site mutation at 456) are capable of converting MTS into an amber-tRNA-binding enzyme.

**The Suppressor MTS Enzymes Recognize the Amber Anticodon.** To evaluate whether the acquisition of a suppressor character by MTS was linked to a broadening of its specificity, tRNA methionylation was assayed using three noncognate tRNAs ($tRNA^{Ile}_{major}$, $tRNA^{Val}_1$, and $tRNA^{Glu}_2$) as substrates of the purified suppressor enzymes WA461DH456-QR211 and DY456. These enzymes were unable to methionylate such tRNAs ($k_{cat}/K_m < 1\ M^{-1}s^{-1}$). Consistent with this, the methionylatable fraction of a crude tRNA preparation was not increased by using the suppressors WA461DH456QR211, WA461DA449AV451QP213, and DY456 or the nonsuppressor WA461 instead of the M547 enzyme.

Interestingly, identical catalytic efficiencies were obtained when the amber derivatives of either $tRNA^{Met}_f$ or $tRNA^{Met}_m$ were used as substrates (Fig. 3). To further demonstrate that suppressor enzymes selectively aminoacylate any tRNA species provided that it has an amber anticodon, strain 121R was lysogenized with one copy of a λ phage expressing amber $tRNA^{Val}_1$ (strain 121R-λvalCUA) and transformed with the pBSM547 vectors encoding M547 mutants. Upon overexpression of the WA461 enzyme, the suppressor phenotype was not observed (suppression efficiency of 0.035 unit). When one of the best suppressor enzymes was used (WA461DA449AV451QP213), the strain became able to grow in the absence of added arginine and the suppression efficiency reached 0.7 unit, corresponding to a 20-fold gain of suppression efficiency. Similarly, a 20-fold gain of suppres-

sion efficiency can be obtained with amber $tRNA^{Ile}$ when expressed in 121R from a multicopy plasmid. Although the amber derivatives of non-Met tRNAs appeared to be less efficient substrates for suppressor MTS than derivatives of the cognate initiator and elongator tRNAs, our results clearly establish that mutations conferring the suppressor phenotype on MTS are indeed directed toward amber anticodon recognition, whatever the tRNA bearing the CUA sequence.

## DISCUSSION

This work further confirms that MTS recognition of the methionine (CAU) anticodon is the major factor in tRNA discrimination. However, the main finding of this study is that both *in vitro* and *in vivo* recognition of tRNA is governed by structural elements of the synthetase located in the 460 region. As already suggested (16, 17), the results indicate that Trp-461 has a crucial role in tRNA binding.

These conclusions are based on the following observations: (*i*) a tRNA must have a CAU anticodon to form a productive complex with MTS; (*ii*) the loss of the aromatic character of residue 461 abolishes complex formation with all tested tRNAs possessing a CAU anticodon; (*iii*) recognition of an amber $tRNA^{Met}$ is conditioned by the occurrence of a point substitution in the 460 region; (*iv*) elimination of Trp-461 is sufficient to improve by several orders of magnitude the frequency of isolation of suppressor MTS *in vivo*.

Interestingly, this region of the protein is located in the C-terminal domain, 40 Å from the active-site crevice formed by the N-terminal domain (28, 29). The localization of the anticodon binding site in the 460 region appears sterically compatible with a proper positioning of the tRNA acceptor arm toward the active site, where it reacts with methionyladenylate (30). In addition, in agreement with the present model, previous experiments showed that the cytosine of the anticodon (C34) could be crosslinked to a single residue, Lys-465, of the MTS sequence (15).

With respect to the restrictive effect of Trp-461 on the frequency of scored suppressor enzymes, it may be that beyond its positive role in the binding of the CAU anticodon, this residue also acts as a negative discriminator toward tRNAs having a non-methionine anticodon. This negative effect exerted by Trp-461 could be overcome by the Asp-456 → Tyr mutation (M547DY456). This change may bring a net gain in discrimination toward the amber tRNAs. However, this gain in discrimination is accompanied by only a slight decrease in the recognition of CAU tRNAs. This favors the idea that the DY456 mutation creates a subsite for an amber anticodon without disrupting the preexisting methionine anticodon site.

The new aminoacylation capacity does not reflect a relaxed specificity, since none of the tested suppressor synthetases (DY456, WA461DA449AV451QP213, or WA461DH456QR-211) has acquired the ability to aminoacylate noncognate tRNAs. It should be emphasized that the suppressor enzymes isolated here have acquired the ability to methionylate amber $tRNA_f^{Met}$ as efficiently as amber $tRNA_m^{Met}$ (Table 2), even though the screening procedure was designed to recognize only the amber $tRNA_m^{Met}$. Moreover, a significant suppression was obtained using amber $tRNA^{Val}$ or $tRNA^{Ile}$ as *in vivo* substrates of the WA461DA449AV451QP213 suppressor MTS. This strongly indicates that the mutations in the 460 region responsible for the suppressor phenotype are indeed directed toward amber anticodon recognition, whatever the rest of the tRNA structure.

Finally, systematic single substitutions of residues in the 460 region [DN456, ED457, EQ457, QH458, PL460, KN465, EQ467, RQ469, DN470, or DN472 (ref. 17) and DY456, EW457, KQ465, or ED467 (this work and ref. 31)] did not change the catalytic efficiency by a factor >8 (results not shown). It is therefore unlikely that the substitutions in this region causing suppression could have acted singly to greatly change the local three-dimensional structure. Though a role for limited structural changes cannot be excluded, it is likely that the identified residues in this region participate directly in the anticodon binding site.

Consistent with the essential role of the 460 region, the amino acid sequence surrounding Trp-461 (residues 452–467) is highly conserved in the sequence of the MTS from *Bacillus stearothermophilus* (unpublished results). We propose that the deciphering of the anticodon sequence by MTS and the identity of this synthetase are specified at least partly by residues 452–467.

It is with sorrow that we mourn the sudden death of Guy Fayat on October 22, 1990. We thank Dr. P. Mellot for help with the initial stage of this study. Dr. S. Brunie and Dr. M. Springer are acknowledged for helpful discussions. This work was supported by grants from the Ministère de la Recherche (87-CO392) and the Fondation pour la Recherche Médicale.

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